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(54) Title: TEST TO DETERMINE AN ORGANISM'S SPECIES AND/OR POPULATION IDENTITY BY DIRECT NUCLEOTIDE SEQUENCE ANALYSIS OF DEFINED SEGMENTS OF ITS GENOME			
(57) Abstract A method is provided for the determination of the genus, and then the species, and/or strain, and/or subspecies and/or subset of an eukaryote or a prokaryote organism, e.g. a mammal, a bird, a reptile, an amphibian, a fish or an invertebrate. The method includes the first step of isolating DNA from the sample. A defined segment of the DNA is then amplified. The nucleotide sequence of the amplified segment is then determined. That DNA sequence is then compared with a data base of DNA sequences from known species. A cladistic analysis of these sequence data is then carried out. A kit is also provided comprising a carrier; compartments within the carrier to receive one container containing DNA from the above described defined organism; a second container containing an amplified segment of the DNA from that defined organism; and a third container containing DNA sequences for a plurality of genera, and species, and/or strain, and/or subspecies and/or subset or DNA derived therefrom.			

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TEST TO DETERMINE AN ORGANISM'S SPECIES AND/OR POPULATION IDENTITY BY DIRECT NUCLEOTIDE SEQUENCE ANALYSIS OF DEFINED SEGMENTS OF ITS GENOME

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(i) Technical Field To Which The Invention Relates

This invention relates to means and methods for the determination of the species of an organism (sample) and/or its population identity by direct nucleotide sequence analysis of defined segments of its genome.

10 (ii) Background Art

Many government agencies require a means of identifying the species origin of meat or other biological specimens. For example, law enforcement agencies need to know if a blood stain comes from a human or an animal. Food inspectors check meat for accurate labelling for economic (e.g. selling cheaper meats, e.g. horse for beef) and religious (e.g. pork for beef) reasons. Wildlife officers must be able to identify the species type of meat to detect animals that have been obtained illegally (i.e. hunted without a permit, out of season, or the quota exceeded). In addition, customs officers require methods for identifying restricted, endangered species or products derived from them. There is therefore, a need for a rapid, accurate and reliable method for determining the species origin of a biological sample.

Currently there are three standard procedures for identifying the animal origin of meat, but each has major drawbacks. The most commonly recommended method involves protein electrophoresis. The soluble proteins are detected by general protein or specific enzyme activity stains after electrophoresis using starch gels, polyacrylamide gels or cellulose acetate strips. This method only works with fresh or frozen tissue. Immunological procedures rely on antibodies raised against soluble proteins. Simple Ouchterlony analyses

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usually suffer from cross-reaction of proteins from related organisms. This procedure may indicate the family of organisms to which the specimen belongs, but rarely can it identify the species. Enzyme-linked immunosorbent assays have been developed that can detect pork or poultry in cooked and canned meat products. However, the polyclonal antibodies raised against the chicken heat-resistant antigens could not discriminate between turkey and chicken products. The third method depends on the ability to separate water-soluble muscle proteins by high-performance liquid chromatography. This procedure has the advantage of being able to estimate the relative proportions of raw meats in mixtures, but it is only applicable to fresh or freshly frozen meats.

The disadvantages of each of the methods outlined above are a consequence of their dependence on the ability to characterise proteins. Many proteins are heat-labile, lose their biological activity soon after death, are subject to modification in different cell types, and their presence is a function of the cell type being examined. It would be preferable to analyze DNA rather than proteins for identifying the species origin of an unknown sample. DNA carries an organism's genetic information. It is the same in all cell types of an animal. Thus, it does not matter if one isolates DNA from a blood stain, a muscle extract, or a liver sample. The information content of DNA is also greater than that of proteins. Information is lost as one goes from DNA to protein because of the degeneracy of the genetic code. Moreover, electrophoretic, immunological, and chromatographic methods do not detect all the amino acid differences that may occur between different proteins (e.g. electrophoresis will only detect changes in the net charge of two proteins and immunological techniques require different antigenic determinants to be present on the proteins under investigation). DNA is a remarkably stable molecule. It has been isolated from animal skins

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from the nineteenth century, mummified tissue and a 7,000 year old human brain.

The keys to identifying different species normally rely heavily on morphological characteristics.

5 However, when an animal has been killed for food or sport, these species-specific markers are often destroyed or removed from the animal. This presents problems for government agencies (e.g., Canadian Department of Fisheries and Oceans, Canadian Wildlife Service, Health 10 and Welfare Canada, and other international bodies) who are involved in determining the species type of the animal or meat to enforce species conservation or health-related regulations. There is thus a great need for a reliable and accurate method for determining the identity 15 of the species of an organism when it is not possible to do so using the normal morphological markers.

Four commercially important tuna species in the genus *Thunnus* are caught commercially off the east coast of Canada. The harvest of bluefin tuna (*T. thynnus*) is 20 regulated, but of bigeye (*T. obesus*), yellowfin (*T. albacares*), or albacore (*T. alalunga*) is not. Enforcement of the regulations governing the bluefin fishery has been difficult because of the close genetic 25 relationships among these species and the ease with which morphological characters may be removed once a fish has been landed. Isoelectric focusing of water-soluble muscle proteins does not resolve these four tuna species beyond two groups: one consisting of bluefin and yellowfin and the other comprising bigeye and albacore.

30 The bluefin tuna (*Thunnus thynnus*) is prized as one of the world's best marine sports fish and commands top prices in the lucrative Japanese sashimi market. The largest member of the family Scombridae, bluefin tuna is one of four species of *Thunnus* that are caught 35 commercially off Canada's east coast. The other tuna species found in this region are big-eye (*T. obesus*), yellowfin (*T. albacares*), and albacore (*T. alalunga*). A

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fear that too many juvenile bluefin tuna were being caught brought about the formation of the International Commission for the Conservation of Atlantic Tunas (ICCAT) in 1969 and regulations governing the minimum size and 5 limiting the total allowable catch of bluefin were introduced in the mid 1970's. The Canadian Department of Fisheries and Oceans has taken additional measures to maintain the bluefin tuna fishery within Canadian waters and these include restricting the number of licences 10 issued, controlling the type of gear used, and limiting the number of bluefin tuna that may be taken per boat per day.

With the value of an individual bluefin tuna ranging from \$1,000 to \$20,000 (1989 Canadian dollars) 15 depending on the size and condition of the fish, it is not surprising that a black market in illegally caught bluefin tuna has arisen. This has made it difficult to maintain the quotas required to conserve and expand the bluefin tuna fishery. Enforcement has also been a 20 problem because it is very difficult to tell a bluefin from a big-eye, yellowfin, or albacore (especially small fish), as all of these species are quite similar in appearance. Moreover, the usual characters used to 25 differentiate between these species are skeletal and visceral in nature (e.g. gillraker counts) and these identifying features are removed once the fish has been landed and gutted.

It is far preferable to examine DNA, the molecule of heredity, rather than proteins. DNA is the same in 30 all cell types of an organism whereas proteins vary from tissue to tissue. DNA is remarkably stable and the amount of information available for analysis decreases as one goes from DNA to protein. This is because of the degeneracy of the genetic code and because protein 35 electrophoresis only detects mutations that change the overall charge of the molecule.

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Some solutions to the problem of establishing the genetic identify of a strain of an animal have been proposed. Some of the tests employed for such determinations rely upon the identification of 5 polymorphic proteins in the plasma, from the surface of cells, or extracted from within the cells of the species in question. However, most of the tests are based on the analysis of a protein or its activity, and so it is the gene product and not the gene itself which is the subject 10 of the investigation. It would therefore be preferable to analyze the gene directly rather than the product of its expression because of the degeneracy that is inherent in the process by which genetic information is expressed.

The flow of genetic information in cells is well 15 known. The information directing the biosynthesis of proteins is encoded in the nucleotide sequences of DNA known as genes. The DNA of the cell may be viewed as the storage form of the genetic information. The DNA molecules are large, chemically-stable, easily replicated 20 and contain many gene sequences.

A problem exists in the isolation and purification of the specific gene or nucleotide sequence containing the genetically coded information for the amino acid sequence of the desired protein. DNA exists 25 in all living cells in the form of extremely high molecular weight chains of nucleotides. A cell may contain more than 10,000 structural genes, coding for the amino acid sequences of over 10,000 specific proteins, each gene having a sequence many hundreds of nucleotides: 30 in length. DNA consists of four different nucleotides: adenine (A); guanine (G); cytosine (C); and thymine (T). The long sequences comprising the structural genes of specific proteins are consequently very similar in overall chemical composition and physical properties. 35 The separation of one such sequence from the plethora of other sequences present in is lated DNA cannot ordinarily

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be accomplished by conventional physical and chemical preparative methods.

In its native configuration, DNA exists in the form of paired linear polynucleotide strands. The strands are usually held together by interactions between an A on one strand, with a T on the opposite strand, and similarly a C on one strand and a G on the other. This is referred to as A-T and G-C base pairing. The complementary base pairing relationships described above exist between the paired strands such that each nucleotide base of one strand exists opposite its complement on the other strand. The entire sequence of one strand is mirrored by a complementary sequence on the other strand. If the strands are separated, it is possible to synthesize a new partner strand, starting from the appropriate precursor monomers (deoxynucleotide triphosphates). The sequence of addition of the monomers starting from one end is determined by, and complementary to, the sequence of the original intact polynucleotide strand, which thus serves as a template for the synthesis of its complementary partner. The synthesis of mRNA corresponding to a specific nucleotide sequence of DNA is understood to follow the same basic principle. Therefore, a specific mRNA molecule will have a sequence complementary to one strand of DNA and identical to the sequence of the opposite DNA strand, in the region transcribed. Enzymic mechanisms exist within living cells which permit the selective transcription of a particular DNA segment containing the nucleotide sequence for a particular protein. Consequently, isolating the mRNA which contains the nucleotide sequence coding for the amino acid sequence of a particular protein is equivalent to the isolation of the same sequence, or gene, from the DNA itself. If the mRNA is retranscribed to form DNA complementary thereto (cDNA), the exact DNA sequence is thereby reconstituted and can, by appropriate techniques, be inserted into the genetic material of

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another organism. The two complementary versions of a given sequence are therefore inter-convertible, and functionally equivalent to each other.

The nucleotide subunits of DNA and RNA are linked together by phosphodiester bonds between the 5' position of one nucleotide sugar and the 3' position of its next neighbour. Reiteration of such linkages produces a linear polynucleotide which has polarity in the sense that one end can be distinguished from the other. The 3' end may have a free 3'-hydroxyl, or the hydroxyl may be substituted with a phosphate or a more complex structure. The same is true of the 5' end. In eucaryotic organisms, i.e. those having a defined nucleus and mitotic apparatus, the synthesis of functional mRNA usually includes the addition of polyadenylic acid to the 3' end of the mRNA. Messenger RNA can therefore be separated from other classes of RNA isolated from an eucaryotic organism by column chromatography on cellulose to which is attached polythymidylic acid.

Transcription is the process by which the retrieval of information is begun. Transcription involves the resynthesis of the information in the form of RNA. Messenger RNA (mRNA) transports the information to the machinery of protein synthesis, the ribosome.

Once the mRNA is synthesized from the gene, the process of protein synthesis may begin. This process is essentially one of molecular decoding, in which the nucleotide sequence of the mRNA provides a template for the synthesis of a particular protein. Since there is a change from a nucleic acid language into that of a protein language, this process of protein synthesis appropriately is referred to as translation. As the mRNA is passed through the ribosome, groups of 3 nucleotides (codons) are positioned such as to orient accessory RNA molecules known as transfer RNA (tRNA), carrying a single amino acid into the proper alignment for the addition of the amino acid to the growing protein chain.

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The coding ratio of nucleotides to amino acids is three nucleotides coding for one amino acid. Since it is necessary to code for twenty different amino acids uniquely with the available four types of nucleotides, 5 three represents the minimum acceptable ratio. A coding ratio of one nucleotide to one amino acid would only accommodate four of the twenty amino acids necessary for protein synthesis. A coding ratio of two yielding 16 (4²) combinations likewise falls short of the required 10 complexity. However, with a coding ratio of three, 64 (4³) different combinations are possible. This excess of twenty code words confers upon the genetic code a conditions known as degeneracy. A degenerate code contains several different code words for the same amino 15 acid. The situation does not exist, however, where one code word would specify two different amino acids. The code may be degenerate, but it is not ambiguous.

The ability to probe the chromosome, extra 20 chromosomal genetic material, messenger, transfer and ribosomal RNA, to synthesize genetic material, as well as to manipulate genetic material, has increased the need for means to analyze the composition and base order of genetic material. It is therefore desirable to provide for recording various genetic fragments which allow for 25 hybridization with the complementary fragment, so that mixtures may be analyzed for the presence or absence of a particular nucleotide sequence. In the development of a system for analyzing for particular nucleotide sequences, there are many considerations. The first 30 consideration is the ability to separate a mixture into its constituent parts, based on molecular weight and/or electrophoretic mobility. The second consideration is the ability to determine accurately the nature of the constituent parts.

35 One method for determining whether a particular sequence exists is hybridization. That is, a particular nucleotide sequence is marked with a detectable label,

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conveniently a radioactive label, and is combined with the nucleotide sequence to be analyzed. If the two sequences hybridize so as to form a strong non-covalent interaction, it may then be reasonable assumed that the 5 sequences are substantially identical. Various techniques for accurately determining whether hybridization has occurred and for qualitatively determining the amount of the nucleotide sequence have been developed.

10 Another method for the detection of specific nucleic acid sequences (DNA or RNA) is achieved by hybridization of radioactive probes and autoradiography. Traditionally, radioactive probes have been produced by nick-translation, and more recently by SP6 transcription. 15 These methods generate radioactive probes of high specific activity which are capable of detecting small concentrations of DNA or RNA sequences. However, there are several disadvantages to these methods. First, the production of probes requires the use of radioactive 20 isotopes which have short half-lives necessitating a continuous production of fresh probes. Secondly, the labelling procedure requires the use of enzymes which are expensive and require reaction conditions which must be very carefully calibrated. Thirdly, radioactive isotopes 25 are biologically dangerous to use. In fact, their use requires proper licensing, and their disposal is becoming increasingly expensive, difficult and hazardous.

A non-radioactive hybridization probe has been developed which utilizes biotinylated nucleotide 30 analogues which are synthesized into probes using procedures described previously for the production of radioactive probes. Hybridization of the probes to the target sequence is detected by the interaction of biotin with avidin-conjugated enzymes, fluorescent compounds, or 35 enzyme-linked immunodetection systems. Although the use of radioactivity is eliminated, several problems are associated with the biotin-avidin technique. One problem

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is sensitivity; for the most part, biotinylated probes are not as sensitive as radioactive probes. Another problem encountered is that the alteration of the nucleotides interferes with hybridization of the probe to 5 its target.

Hybridization assays using various signal generating systems have also been developed. For example, a non-radioactive hybridization assay system which utilizes the chemical modification of nucleic acids 10 that makes them more immunogenic has been developed. The signal is generated by reporter groups containing antibodies which recognize the altered nucleic acid. In another hybridization system, the adenine and cytosine nucleic acid bases of the probe are bonded to reporter 15 groups by chemical modification.

The hybridization assays thus far described require the preparation of labelled probes, each of which hybridizes to a different target sequence. In order to reduce the number of labelled probes that must be 20 prepared, a hybridization assay has been developed which utilizes a "bridging" polynucleotide between the target nucleic acid and a general signalling polynucleotide. The bridging polynucleotide consists of a single-stranded filamentous bacteriophage which contains nucleic acid 25 sequences complementary to the target gene. The general signalling polynucleotide is a segment of single strand phage DNA which is complementary to the bridging polynucleotide.

Another problem associated with hybridization 30 assays involves the removal of labelled probes that do not hybridize to the target sequence from the hybridization reaction because the presence of non-hybridized probes in the hybridization reaction leads to a false positive result. A hybridization assay has been 35 provided which claims to obviate the requirement for separation of non-hybridized probes. This system requires the cohybridization of two components which,

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when associated, generate a signal. Another hybridization assay has been provided which utilizes photochemically-reactive intercalating agents for the covalent attachment of nucleic acids to solid supports.

5 The immobilized target nucleic acids are capable of hybridization. Yet another immobilized hybridization assay system first involves hybridizing and then forming covalent bonds between the probe and target sequence. Immobilized sandwich hybridization techniques which

10 require two distinct single-stranded nucleotide probes have also been reported.

Knowing the sequence of nucleotides of a messenger RNA, it is possible to write explicitly the sequence of amino acids coded therein, but the reverse is

15 not true. Because of the degeneracy of the genetic code, a number of nucleotide sequences would be consistent with a given amino acid sequence.

In the scheme of dividing organisms with respect to the internal architecture of the cell, all cellular

20 organisms are either prokaryotic or eukaryotic. Prokaryotes are less complex than eukaryotes in that they lack internal compartmentalization by unit membrane systems and lack a defined nucleus. Prokaryotic genetic information is carried in the cytoplasm on double-stranded, circular DNA; no other DNA is present in these

25 cells (except for the possible presence of phage, bacterial viruses, and circular DNA plasmids, capable of autonomous replication). Eukaryotes on the other hand have a multiplicity of unit membrane systems which serve

30 to segregate many of the functional components into specialized and isolated regions. For example, molecules storing genetic information (DNA) can be found in a well-compartmentalized nucleus and also in organelles; mitochondria and (in photosynthetic organisms)

35 chloroplasts. The replication, transcription and translation of the eukaryotic genome occurs at either two or three distinct sites within the cell: in the

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nucleocytoplasmic region, in the mitochondrion and in the chloroplast.

The differences between prokaryotes and eukaryotes, however, break down when a comparison of 5 mitochondria and chloroplasts is carried out with prokaryotes: these organelles are today considered to have been derived from free-living prokaryotes, which entered into an endosymbiotic relationship with primitive eukaryotes, and eventually became closely integrated with 10 the machinery of the host cell and incapable of independent existence.

In this model, the eukaryotic cell is a phylogenetic "chimera" with organelle components that are clearly prokaryotic in nature. The "prokaryotic-eukaryotic" dichotomy then also has drawbacks, even as a 15 broad classification method.

Where classification of organisms becomes more than a scientific exercise is in the identification of animals for breeding purposes. For example, the cattle 20 breeder, or fish breeder, may wish to have a quick and reliable means of identifying different species and strains of their subjects. The correct identification of the species of these organisms is of particular importance.

25 A molecular approach to bacterial classification is to compare two genomes by DNA-DNA reassociation. A genetic definition of species includes the provision that strains of species are 70% or more related. With DNA-DNA reassociation, a strain can be identified only if the 30 radioactively labelled DNA probe and unknown DNA are from the same species. The practical application of this 70% species definition, however, is limited by selection of an appropriate probe. This may be overcome in part by selecting phenotypic attributes which seem to correlate 35 with the reassociation group; but when these are used alone, the DNA-DNA reassociation species definition is also applied indirectly. Restriction endonuclease

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analysis can therefore be carried out to determine common sequences in isolated genes.

The main limitation of present gene detecting methods is that they are not sensitive enough and therefore require a relatively large amount of sample to verify accurately the existence of a particular gene sequence. This is not surprising since the detection of a single gene in the entire genetic repertoire of a human being requires locating one part in one to ten million. In fact, most hybridization methods require at least one to ten micrograms of purified DNA, representing a substantial sample of cells, to perform a reliable analysis. This limitation is particularly significant in prenatal diagnosis of genetic disorders where only a small cell sample can be taken, or in identifying infectious agents such as viruses in small tissue samples. Consequently, there is a substantial need for gene detection methods which are more sensitive than the hybridization assay and which provide more information.

A series of similar individuals showing certain common features that are recognizably different from other such series is referred to as a species. Thus, members of a particular species may be identified because they exhibit certain defined characteristics or traits.

Although these characteristics are usually morphological, there is an underlying genetic basis for them. The traits are maintained by the genetic structure of the species. Members of the species can breed successfully with other members of the species, but do not do so with members of other species. This means that individuals within a species will share a specific set of genetic markers that may be used to define that particular species.

There is also variation among members of species. This variation may be restricted to a single individual and its immediate relatives, or it may be characteristic of a subset of the species as a whole. A subset may

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constitute a subspecies or population (i.e. a group of individuals within a species who confine their breeding to other members of that group although they are capable of breeding with any member of the species). The sum of 5 the genetic content of all the populations in a species forms the gene pool of that species. Therefore, the genetic material of an organism should comprise: (a) segments that are unique to themselves (individual identifiers); (b) segments that are shared by other 10 individuals that are part of its subspecies or population (population identifiers); (c) segments that are found in every member of the species but not in other related species (species identifiers); and (d) components that are found in many different related species. By 15 judicious use of these different regions of a species' genetic material, it should be possible to determine the species and/or population origin of an individual.

There is a long history of using protein markers for identifying different species. However, proteins 20 have several shortcomings in this respect. For example, they vary from tissue to tissue and therefore cannot be used interchangeably when one has samples of hair, skin, blood and muscle to examine. Changes to proteins may occur after death or during food-processing (e.g. even 25 upon freezing and thawing). In addition, there is a loss of information content as one goes from DNA to protein and it is not always possible to use proteins to tell closely related species apart.

Identity is generally defined as "sameness in all 30 that constitutes the objective reality of a thing". Though the primary concern is with the identification of the species and/or population of an organism, the problem of how best to carry out identification arises in those 35 situations in which one must determine whether an unknown object, in all essential respects, matches a particular known object lying within a large collection of diverse known objects. In the context of biology, the term

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id ntification, as used herein, encompasses the determination of whether an unknown organism, or tissue derived therefrom, falls within a particular group of organisms constituting a species or a subset of that 5 species, i.e. a population.

The logical approach to the problem of identification is to find an identifier or identifiers that are shared by all members of a species and/or population but are absent from other species. The 10 genetic information, or DNA, of an individual contains segments that can be used as species and/or population identifiers.

Canadian Patent No. 1,215,304, patented December 16, 1986 by J. Glassberg, attempted to solve one 15 particular problem relating to the determination of paternity in sexually reproducing organisms and to establish individual genetic identity. These objectives were said to be achieved by analyzing the DNA of the organism in respect to one or more polymorphic genetic 20 regions, differentiating the polymorphisms in terms of relative size of the genetic regions and by so doing, characterize an individual member of the species. It was also taught that the polymorphic genetic regions could be detected by the following steps: isolating the DNA of 25 the individual to be analyzed; subjecting that DNA to the action of restriction endonucleases; sizing and converting DNA fragments generated as described above to single-stranded molecules; hybridizing the sized, single-stranded molecules with probe DNA molecules and 30 identifying the number and location of the hybridized fragments. The probe molecules were those which had been generated by endonuclease digestion of genomic DNA.

It was also taught, by that patentee, that the 35 polymorphic genetic regions could be detected by the following steps: isolating the DNA of the individual to be analyzed; subjecting the DNA to the action of restriction endonucleases; sizing and converting DNA

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fragments generated as described above to single-stranded molecules; hybridizing the sized, single-stranded molecules with probe DNA molecules; and identifying the number and location of the hybridized fragments. The 5 probe was one that did not hybridize to the human HLA genetic locus.

However, this technique did not provide for the determination of the identify of the genus, and within a particular genus, the identify of the species and/or 10 strain and/or subset and/or sub species of that particular species of an organism (sample) and/or the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes.

15 Canadian Patent No. 1,214,980, patented December 9, 1986 by J.A. Webster, Jr., was alleged to provide a method of objectively identifying organisms which utilized the organisms' genomes. The patent provided a method of characterizing an unknown organism which 20 comprised comparing the electrophoretic pattern of restriction endonuclease-digested DNA from the organism, which digested DNA had been hybridized or reassociated with ribosomal RNA information-containing nucleic acid from or derived from a probe organism, with equivalent 25 electrophoretic patterns of at least two known different organism species. Thus, the patentee specifically provided a method of characterizing an unknown organism by the steps of: comparing the electrophoretic pattern of restriction endonuclease-digested DNA from an unknown 30 organism, the digested DNA having been hybridized or reassociated with ribosomal RNA information-containing nucleic acid from or derived from a known probe organism, with at least two equivalent electrophoretic patterns, each one of the equivalent electrophoretic patterns 35 defining a known different organism species; and establishing the species of the unknown organism by means of a conserved set of ribosomal RNA sequence-containing

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restriction fragments present in the electrophoretic pattern of the unknown organism.

That patentee also provided a method for detecting a prokaryotic organism while in the presence of, or associated with, a eukaryotic organism which comprised: selectively hybridizing ribosomal RNA sequences of the prokaryotic organism with a detectably-labelled prokaryotic rRNA information containing hybridization probe.

That patentee still further provided a kit comprising a carrier: compartment in the carrier to receive in close confinement therein one or more containers, a first container containing ribosomal RNA (rRNA) information-containing nucleic acid from, or derived from, a probe organism; and a catalogue having electrophoretic band patterns of rRNA information-containing restriction fragments for at least two known different organism species, or DNA derived therefrom. The kit could also include a second container containing one or more restriction endonuclease enzymes.

However, this technique did not provide for the determination of the identify of the genus, and within a particular genus, the identify of the species and/or strain and/or subset and/or sub species of that particular species of an organism (sample) and/or the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes.

Canadian Patent No. 1,221,298, patented May 5, 1987 by J.A. Webster, Jr., provided a method of characterizing an unknown organism species by determining the position of part or whole of evolutionarily-conserved sequences in genetic material of the organism, relative to a known position of restriction endonuclease cleavage sites in the genetic material (other than by determining the electrophoretic pattern of restriction endonuclease digested DNA from the unknown organism, which digested

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DNA has been hybridized or reassociated with ribosomal RNA information-containing nucleic acid from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of the unknown 5 organism, and then comparing that characterization with information from at least two sets of identifying genetic characterizations derived from the same conserved sequences, each of the sets defining a known organism species.

10 That patent also provided a kit comprising: a carrier; compartments therein to receive in close confinement therein one or more containers, in which a first container contained conserved genetic material sequence information-containing nucleic acid (other than 15 ribosomal RNA information-containing nucleic acid) from, or derived from, a probe organism or from a consensus sequence; and containing a catalogue having hybridized or reassociated electrophoretic band patterns for at least two known different organism species, or genetic 20 material derived therefrom. That kit could also include a second container containing one or more restriction endonuclease enzymes.

However, this technique did not provide for the determination of the identify of the genus, and within a 25 particular genus, the identify of the species and/or strain and/or subset and/or sub species of that particular species of an organism (sample) and/or the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of 30 genomes.

U.S. Patent No. 4,283,489, patented August 11, 1981 by H.M. Goodman et al., provided a method for purifying a specifically desired DNA sequence, starting from RNA heterogeneous in length and sequence. The 35 method employed restriction endonuclease cleavage of cDNA transcribed from a complex mixture of mRNA. The method made use of transcription of RNA into cDNA, the sequence

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specific fragmentation of this cDNA with one or two restriction endonucleases, and the fractionation of the cDNA restriction fragments on the basis of their length. The use of restriction endonucleases eliminated size 5 heterogeneity and produced homogeneous length DNA fragments of desired sequences. The length of the fragments depended on the number of nucleotides separating the restriction sites and will usually be different for different regions of DNA. Fractionation by 10 length enabled purification of a homogeneous population of fragments having the desired sequence. The fragments were homogeneous in size and highly pure in terms of nucleotide sequence.

Specific sequences purified by the procedure 15 outlined above could be further purified by a second specific cleavage with a restriction endonuclease capable of cleaving the desired sequence at an internal site. This cleavage resulted in formation of two sub-fragments 20 of the desired sequence, separable on the basis of their length. The sub-fragments were separated from uncleaved and specifically cleaved contaminating sequences having substantially the same original size. Because of the 25 rarity and randomness of placement of restriction endonuclease recognition sites, an extremely low probability that a contaminant having the same original length will be cleaved by the same enzyme to yield fragments having the same length as those yielded by the desired sequence. After separation from the 30 contaminants, the sub-fragments of the desired sequence may be rejoined using techniques known in the art, to reconstitute the original sequence. The two sub-fragments must be prevented from joining together in the reverse order to their original sequence.

However, this technique did not provide for the 35 determination of the identify of the genus, and within a particular genus, the identify of the species and/or strain and/or subset and/or sub species of that

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particular species of an organism (sample) and/or the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes.

5 U.S. Patent No. 4,302,204, patented November 24, 1981 by G.M. Wahl et al., provided techniques for the transfer and detection of separated nucleic acids, both RNA and DNA. The patentee provided for the preparation of resolved nucleotide sequences covalently affixed to a
10 stable substrate, which are then used for hybridization with nucleotide sequences for determination of the presence of a complementary nucleotide sequence. In an initial phase, a particular nucleotide sequence was prepared for transfer from a source of the sequence to a
15 chemically-reactive substrate, e.g. diazo substituted paper, to affix the nucleotide sequence to the substrate by covalent bonding of the chemically reactive functionality to the polynucleotide, to provide for storage stability. When the nucleotide sequence had been
20 previously subjected to resolution, particularly a resolution based on molecular weight and electrophoretic mobility, the position of the nucleotide sequence on the paper was related to its chemical composition and molecular weight. Once the nucleotide sequence was
25 transferred from the resolving medium to the substrate, and covalently affixed thereto, the substrate may now be used for probing compositions having unknown nucleotide sequences to determine the presence of a sequence complementary to the affixed nucleotide sequence. A
30 hybridization buffer was employed including a volume exclusion or renaturing agent, which greatly enhances the rate and efficiency at which a complementary nucleotide strand hybridizes to the affixed strand.

Prior to hybridization, DNA was treated
35 differently from RNA. Depending upon the molecular weight of the DNA during electrophoresis, differing mixtures are employed to enhance resolution. Where

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relatively low molecular weight DNA was involved, cross-linked polymers were employed to provide for a hard polymer, where the cross-links therein were susceptible to cleavage without adverse affects on the DNA. Where 5 the DNA was of a size in excess of 200 bases in length, the DNA was subjected to successive treatments of depurination and then, degradation and denaturation, so as to provide for randomly-formed single-stranded smaller fragments. The nucleotide sequences to be assayed may be 10 labelled, particularly with a radioactive marker. After hybridization, the presence of the radioactively-marked nucleotide sequences may be determined by autoradiography. In this manner, the presence or absence 15 of a particular sequence can be determined, as well as a quantitative evaluation of its amount.

However, this technique did not provide for the determination of the identify of the genus, and within a particular genus, the identify of the species and/or strain and/or subset and/or sub species of that 20 particular species of an organism (sample) and/or the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes.

U.S. Patent No. 4,358,535, patented November 9, 25 1982 by S. Falkow et al., provided specific DNA probes for use in diagnostic microbiology. In carrying out the patented method, a clinical isolate suspected of containing the pathogen may be used directly or cultivated under conditions where clones are grown 30 providing high multiplication of the pathogenic organism. After treating the genome to provide single-stranded genomic nucleic acid and fixing the nucleic acid to a support, the affixed DNA or RNA was contacted with a labelled polynucleotide having a base sequence 35 complementary to the coding or antisense strand of a gene coding for a product characteristic of the pathogen.

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The primary reagent was the labelled probe. The probe may be RNA or DNA. The probe will normally have at least 25 bases, more usually at least 30 bases, and may have up to 10,000 bases or more, usually having not more than 5,000 bases. The probe sequence will be at least substantially complementary to a gene coding for a product characteristic of the pathogen, usually a cytoplasmic product or released product, particularly an excreted product. The probe need not have perfect complementarity to the sequence to which it hybridizes; there may be 30% or more of mismatched pairs.

However, this technique did not provide for the determination of the identify of the genus, and within a particular genus, the identify of the species and/or strain and/or subset and/or sub species of that particular species of an organism (sample) and/or the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes.

U.S. Patent No. 4,526,865, patented July 2, 1985 by R. Silman, provided a microorganism identification technique. The patented method for identifying an unknown microorganism included the steps of: preparing a specimen of the microorganism to which a radioactive emissive agent has been added that is actively incorporated into the products of metabolism of the microorganism to produce a mix of radioactive peptide or protein-emissive products in a manner that depends on the metabolic mechanism of the microorganism; separating the peptide or protein emissive products in the mix resulting from the preparation of a specimen; detecting the separated peptide or protein-emissive products to derive a characteristic pattern therefrom that depends on the conditions under which the specimen is prepared, which conditions are standardized to provide a repeatable characteristic pattern functioning as a unique identifier for the unknown microorganism; and comparing the

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identifier for the unknown microorganism with stored information relating to the characteristic patterns of known microorganisms to determine the identification of the unknown.

5 However, this technique did not provide for the determination of the identify of the genus, and within a particular genus, the identify of the species and/or strain and/or subset and/or sub species of that particular species of an organism (sample) and/or the
10 population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes.

U.S. Patent No. 4,882,269, patented November 21, 1989 by R.J. Schneider et al., provided an amplified
15 hybridization assay. The patented invention provided a hybridization assay that generated an enormously amplified signal upon hybridization to the target sequence of interest. Accordingly, a primary probe was provided, a small segment of which hybridized to the
20 target DNA of interest. A family of signal-generating secondary probes was also provided which provided for an enormous amplification of the signal generated by the hybridization event. Depending upon the configuration of the assay components, the hybridization event may occur
25 between mobile species or between combinations of fixed and mobile species. Thus, the patentee provided a method for the detection of a target nucleotide sequence, comprising: contacting the target nucleotide under conditions that permit hybridization with a primary probe
30 which comprised a polynucleotide sequence and a polymeric tail that had binding sites that were incapable of binding to the target sequence, and a plurality of secondary probes comprising a family of signal-generating probes, each member of which comprised a signal-generating component and a polymer capable of binding to a different portion of the tail of the primary probe; and
35 detecting the amplified signal generated by a previously-

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formed reaction product, in which the polynucleotide sequence of the primary probe was hybridized to the target nucleotide and a plurality of secondary probes were bound to different portions of the primary probe tail. The patentee also provided a hybridization assay kit for the detection of a target nucleotide sequence, comprising: a primary probe which comprised a polynucleotide sequence that is complementary to the target nucleotide sequence and a polymeric tail that had binding sites that were incapable of binding to the target sequence; and a plurality of secondary probes comprising a family of signal-generating probes, each member of which comprised a signal-generating component and polymer capable of binding to a different portion of the tail of the primary probe, which provided for the generation of an amplified signal when the polynucleotide sequence of the primary probe is hybridized to the target nucleotide and the plurality of secondary probes are bound to a different portion of the primary probe tail.

However, this technique did not provide for the determination of the identify of the genus, and within a particular genus, the identify of the species and/or strain and/or subset and/or sub species of that particular species of an organism (sample) and/or the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes.

U.S. Patent No. 4,908,307, patented March 13, 1990 by K.D. Rodland et al., provide a hybridization probe and method for detecting nucleotide sequences. Initially, a DNA sample of interest was purified chemically and cut into pieces with a suitable restriction enzyme. The pieces were separated by size by electrophoresis in a suitable gel. The pieces of interest were then typically transferred to an immobilizing medium, e.g. a nitrocellulose or nylon-base membrane, that retained the geometry of the pieces. The

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membrane was thereafter dried and prehybridized to equilibrate it for later immersion in a hybridization solution.

A probe was constructed from a nucleotide sequence complementary to the gene sample by a nick translation reaction, using both a DNase and DNA polymerase. The probe and sample are thereafter combined in a hybridization buffer and incubated. In such buffer, the probe and sample are combined in the absence of reducing agents, nonpolar solvents and dextran sulfate. After a specified incubation period, the membrane is removed from the buffer and washed free of extraneous materials. The presence or absence of the particular nucleotide sequence is detected by autoradiography and quantified by liquid scintillation counting.

However, this technique did not provide for the determination of the identify of the genus, and within a particular genus, the identify of the species and/or strain and/or subset and/or sub species of that particular species of an organism (sample) and/or the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes.

Mullis et al., U.S. Patent No. 4,683,195 patented July 28, 1987 provided a teaching of the polymerase chain reaction (PCR) process for amplifying, detecting, and/or cloning nucleic acid sequences. According to the patent, the process comprised treating separate complementary strands of the nucleic acid with a molar excess of two oligonucleotide primers, extending the primers to form complementary primer extension products which acted as templates for synthesizing the desired nucleic acid sequence, and detecting the sequence so amplified. The steps of the reaction could be carried out stepwise or simultaneously and could be repeated as often as desired.

In addition, a specific nucleic acid sequence could be cloned into a vector by using primers to amplify

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the sequence, which contained restriction sites on the non-complementary ends, and a nucleic acid fragment could be prepared from an existing shorter fragment using the amplification process. A complete copy of this patent is 5 attached to this patent application. The entire content of this patent is incorporated herein by reference.

By using the PCR process, unique sequences could be cloned in vitro in a matter of hours. Furthermore, the procedure was easily automated, so that hundreds of 10 samples could be amplified each day. The enzymatic amplification of a specific DNA segment was made possible by the highly specific binding of oligonucleotide primers to sequences flanking the segment. These primers allowed the binding of a DNA polymerase that then copied the 15 segment. Because each newly made copy can serve as a template for further duplication, the number of copies of the target segment grew exponentially.

A description of the polymerase chain reaction was also given in the October 1, 1990 issue of "Chemical 20 and Engineering News". As described therein, PCR was an enzymatic process that was carried out in discrete cycles of amplification, each of which could double the amount of target DNA in the sample. Thus, n cycles can produce 2^n times as much target as was present to begin with.

It was not necessary to know the nucleotide sequence of a target in order to amplify it using PCR, but the sequence of a small stretch of nucleotides on each side of the target must be known. These flanking sequences were used to design two synthetic single-stranded 30 oligonucleotides, usually 20 nucleotides in length, that could serve as primers. The sequence of these primers was chosen so that each had base pair complementarity with its respective flanking sequence.

PCR begins by denaturing the double-stranded 35 target DNA, followed by annealing the primers (one for each strand) to the sequences flanking the target. Each primer formed a duplex with its flanking sequence so that

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th 3'hydroxyl end of the primer faced the target sequence. Addition of DNA polymerase and deoxynucleoside triphosphates caused a new DNA strand to form beginning at the primer and extending across the target sequence, 5 thereby making copies of the target. These steps - DNA denaturation, primer annealing, and DNA polymerase extension - represent one PCR cycle; each step was carried out at an appropriate temperature.

If the extension product of a primer was long 10 enough, it would include the sequence complementary to the primer at the other end of the target sequence. Thus, each new extension product could act as a template for the next cycle. It is this fact, first recognized by Mullis, that lead to the exponential increase in PCR 15 product with each cycle. If the amount of target exactly doubles with each cycle, as few as 20 cycles will generate about a million times more target sequence than originally was present.

Amplification by PCR was extremely rapid. 20 Twenty-five cycles could be carried out in just over one hour. Beginning with 1 μ g of total human DNA, which contained 300,000 copies of each unique sequence, 25 cycles of PCR could generate up to several micrograms of a specific product (on the order of a few picomoles). 25 The size of the final product was equal to the sum of the lengths of the two primers and the length of the target sequence that stretched between them. PCR products up to several thousand base pairs long could be routinely synthesized.

30 The scientific literature has also provided some disclosure relevant to some extent to the present invention.

Meyer et al., "MITOCHONDRIAL GENES AMPLIFIED VIA THE POLYMERASE CHAIN REACTION INFERENCES FOR THE 35 EVOLUTION OF CICHLID FISHES MITOCHONDRIAL GENE SEQUENCES". Annual Meeting of the American Society of Zoologists, American Microscopical Society, Animal

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Behaviour Society, The Crustacean Society, International Association of Astacology Society of Systematic Zoology, and the Western Society of Naturalists, San Francisco, California, U.S.A., December 27-30, 1988. AM ZOOL 28 5 (4). 1988. 35A. merely described the use of PCR/direct sequencing to analyze the genetic relationships among a group of fish. According to this paper, a fast method for sequencing of DNA was developed and applied to mitochondrial genes of cichlids. Parts of the 12S rRNA 10 and the cytochrome-b gene were amplified via the polymerase chain reaction (PCR). The amplification products were sequenced directly by the dideoxy technique. A total of 20 taxa from Africa and the Neotropics were examined and their phylogenetic 15 relationships determined. The rate of sequence divergence was found to be about twice as fast in the cytochrome-b as in the 12S rRNA gene.

KOCHER et al., "AMPLIFICATION AND DIRECT SEQUENCING OF DIVERGENT MITOCHONDRIAL DNAs VIA THE 20 POLYMERASE CHAIN REACTION". Colloquium On Molecular Evolution held at the 18th Annual UCLA (University of California-Los Angeles) Symposia On Molecular and Cellular Biology, Lake Tahoe, California, US, February 27-March 6, 1989. J Cell Biochem Suppl 0 (13 part c). 25 1989. 120, taught that primers that amplified three regions of human mitochondrial DNA via the polymerase chain reaction also amplified the corresponding regions from 99 other species, including mammals, birds, amphibians, fishes, and some invertebrates. 30 Amplification and direct sequencing were possible using unpurified mtDNA from nanogram samples of fresh specimens and microgram amounts of tissues preserved for months in alcohol or decades in the dry state. These sequences provided a consistent metric for phylogenetic comparisons 35 ranging from the population to the interclass level in vertebrates.

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Meyer et al., "MITOCHONDRIAL GENES AMPLIFIED VIA THE POLYMERASE CHAIN REACTION DYNAMICS OF NUCLEOTIDE SUBSTITUTIONS AND EVOLUTION OF CICHLID FISHES", Colloquium On Molecular Evolution held at the 18th Annual 5 UCLA (University Of California-Los Angeles) Symposia On Molecular and Cellular Biology, Lake Tahoe, California, USA, February 27-March 6, 1989. J Cell Biochem Suppl 0 (13 part c). 1989. 132, taught that parts of the mitochondrial 12S rRNA and the cytochrome-b genes of 10 cichlid fishes could be amplified with human primers and sequenced directly. 20 taxa of cichlid fishes from Africa and the Neotropics were examined and their phylogenetic relationships investigated. Sequence divergence was found to be about twice as fast in the 15 cytochrome-b as in the 12S rRNA gene. The evolutionary patterns of sequence divergence was found to be the same in these morphologically fast evolving fishes, as it was for published cases of mammals. In the cytochrome-b gene, it was found that there was a strong transition 20 bias for closely related taxa and C-T changes outnumbered G-A by far. Replacement changes were rare, amino acid changes matched predictions based on the function of cytochrome-b. The 12S rRNA sequences diverged at faster rates in loops than in stem regions.

Kocher et al., "DYNAMICS OF MITOCHONDRIAL DNA EVOLUTION IN ANIMALS AMPLIFICATION AND SEQUENCING WITH CONSERVED PRIMERS", Proc Natl Acad Sci USA 86 (16). 1989. 6196-6200, described the use of the polymerase chain reaction to amplify homologous segments of mtDNA from 30 more than 100 animal species, including mammals, birds, amphibians, fishes, and some invertebrates using a standard set of primers directed toward conserved regions. Amplification and direct sequencing were possible using unpurified mtDNA from nanogram samples of 35 fresh specimens and microgram amounts of tissues preserved for months in alcohol or decades in the dry state. The bird and fish sequences were found to have

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evolved with the same strong bias toward transitions that holds for mammals. Amino acid replacement in a segment of the cytochrome-b gene was found to be faster in mammals and birds than in fishes and the pattern of 5 replacement fit the structural hypothesis for cytochrome-b. Thus, the paper described methods and primers, the method being described to obtain the sequences from known specimens for evolutionary studies. The intent of the authors was an unstated implication that if one knew the 10 sequence one could determine the species.

Jayaraman et al., "PCR MEDICATED GENE SYNTHESIS" Nucleic Acids Res. 1989 Volume: 17 Number: 11, page: 4403, described a method for gene synthesis which involved a combination of a single-step ligation of 15 oligonucleotides and PCR amplification of the crude ligation mixture. This procedure enabled the synthesis of tuna and horse cytochrome C genes.

Whitmore et al., "RFLP ANALYSIS OF FISH MITOCHONDRIAL DNA AMPLIFIED BY THE POLYMERASE CHAIN 20 REACTION", Annual Meeting of the American Society of Zoologists, American Microscopical Society, Animal Behaviour Society, the Crustacean Society, International Association of Astacology, and the Society of Systematic Zoology, San Antonio, Texas, USA, December 27-30, 1990. 25 Am Zool 30(4), 1990, 72A described non-invasive genetic analysis of fish mitochondrial DNA genes, which performed by isolating DNA from the epidermis covering a few scales. The polymerase chain reaction (PCR) was used to amplify selected sequences, including portions of the 12S 30 rRNA, cytochrome-b and ATPase 6 genes. Interspecific and intraspecific variation in several centrarchid species was examined both by restriction endonuclease digestion of the PCR products and allele-specific oligonucleotide probes (ASOPs). Species were readily identified by RFLP 35 profiles of amplified sequences. Maternal lineage of hybrids could also be readily deduced from RFLP profiles. This paper thus described a method using PCR/direct

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sequencing to identify fish species. No details were given, but it could be inferred that the methodology was an extension of the Kocher et al. papers described above.

Block et al., "EVOLUTION OF ENDOTHERMY IN 5 SCOMBROID FISHES: SYSTEMATIC RELATIONSHIPS EXAMINED WITH DIRECT SEQUENCING OF MITOCHONDRIAL DNA". Annual Meeting Of The American Society of Zoologists, American Microscopical Society, Animal Behaviour Society, The Crustacean Society, International Association of 10 Astacology, And The Society of Systematic Zoology, San Antonio, Texas, USA, December 27-30, 1990. Am Zool 30 (4). 1990. 72A, described the relationships of the five families of fishes comprising the suborder Scombroidei using mtDNA. This paper described the amplification and 15 sequencing of the mitochondrial cytochrome-b in Scombrids (tunas). They looked at 15 taxa (no species names given). No sequence data or methods are described.

Carr and Marshall, "DETECTION OF INTRASPECIFIC 20 DNA-SEQUENCE VARIATION IN THE MITOCHONDRIAL CYTOCHROME-B GENE OF ATLANTIC COD (GADUS-MORHUA) BY THE POLYMERASE CHAIN-REACTION". Canadian Journal of Fisheries and Aquatic Sciences, 1991, V48, N1 Pages 48-52, described the determination of the DNA sequence of a portion of the mitochondrial cytochrome-b gene for 55 Atlantic cod 25 (Gadus morhua) from Norway and from ten locations within the Northern Cod complex and adjacent stocks off Newfoundland. DNA was prepared for sequencing by the polymerase chain reaction (PCR). This paper thus described using PCR/direct sequencing to look at the 30 population structure of cod stocks. It did not use the technique for species identification.

A description of the present invention has been published after the priority date of the present application in a paper by BARTLETT and DAVIDSON, 35 "IDENTIFICATION OF THUNNUS TUNA SPECIES BY THE POLYMERASE CHAIN-REACTION AND DIRECT SEQUENCE-ANALYSIS OF THEIR MITOCHONDRIAL CYTOCHROME-B GENES" in Canadian Journal of

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Fisheries and Aquatic Sciences, 1991, V48, N2, P309-317. Thus, that paper described the forerunner of the present invention and has been incorporated into the present specification.

5 (iii) Disclosure of the Invention

The technical problem to be solved is that the use of PCR technique in the prior art as discussed above did not provide for the determination of the species' identity of the species of an organism (sample) and/or 10 the population identity of that organism (sample) by being able to define the nucleotide sequences of segments of genomes. Accordingly, there presently exists a need for a rapid, accurate and reliable means for identifying species and subspecies. Further, a need also exists for 15 a method useful for determining the genus, species, strain, sub-species, sub-sets or population origin of an individual organism or tissue derived from that organism. The method should be generally and readily useful in diagnostic laboratories. It should not be dependent on 20 the number of tests done, on the subject prejudices of the persons carrying out the tests, nor the fortuitous or unfortuitous trial and error methods of the past.

Thus the technical problem to be solved is to provide a method which can quickly, reliably, accurately 25 and objectively determine the genus, species, strain, sub-species, sub-sets or population identity of an organism or tissue derived from that organism by means which utilizes the organism's genome. This technical problem may also be characterized as being to provide a 30 method of analysis to define segments of genomes which may be used to determine genus, species, strain, sub-species, sub-sets or population identity.

The present invention generally provides a method which examines the genetic material itself: i.e. the DNA 35 directly. DNA is identical in every cell type in an individual. It is chemically more stable than proteins and is still accessible for analysis even after several

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food processes (e.g. smoking, salting, pickling, canning). The whole repertoire of genetic identifiers, from individual markers to species specific markers is available by choosing appropriate segments of the genome 5 for analysis.

The method of this invention is for the determination of the genus, and then the species, and/or strain, and/or sub species and/or sub-set of a sample of an organism, whether it is eukaryote or prokaryote, e.g. 10 a mammal, a bird, a reptile, an amphibian, a fish or an invertebrate. The method is characterized by the steps of: isolating DNA from the sample; amplifying a defined segment of that DNA; determining the nucleotide sequence of that amplified segment; comparing that DNA sequence 15 with a data base of DNA sequences from known species; and carrying out a cladistic analysis of these sequence data; thereby to determine the identity of the sample.

This invention also provides a kit comprising a carrier; compartments therein to receive at least one 20 container containing DNA from a defined organism, whether it is eukaryote or prokaryote, e.g. a mammal, a bird, a reptile, an amphibian, a fish or an invertebrate; another container containing an amplified segment of the DNA from that defined organism; and another container containing 25 DNA sequences for a plurality of genera, and the species, and/or strain, and/or sub species and/or sub-set of DNA derived therefrom.

The advantageous effect of this invention is that it provides a procedure for identifying the animal origin 30 of biological specimens, namely, Forensically Informative Nucleotide Sequencing (FINS). FINS is composed of four steps. FINS is used to identify the species of origin of a specimen, according to this invention, by providing conditions that allow each step to be carried out 35 routinely and reproducibly. The steps are as follows: first, DNA is extracted from the biological sample; second, a specific segment of the isolated DNA is

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amplified; third, the nucleotide sequence of the amplified DNA is determined; and fourth, a phylogenetic analysis is carried out on the nucleotide sequence to determine its closest relative(s) in a data base.

5 The advantageous effect of this invention, relies on the ability to isolate DNA from the sample in question; the amplification of a defined segment of the DNA; the determination of the DNA sequence of the amplified segment; the comparison of this DNA sequence 10 with a data base of sequences from known species; and a cladistic analysis of these sequence data.

15 The method of one mode of this invention is characterized by the steps of first determining if there is a match of the DNA sequence of a sample with any DNA sequence in the data base. If no direct match is observed, a cladistic analysis is carried out to determine the closest genus, species, strain, sub-species, or sub-sets. DNA is extracted from these genera, species, strains, sub-species, or sub-sets. The 20 segments of interest are amplified and sequenced and then compared with the sequence from the sample. These steps are repeated until a match is found and the identity of the sample established. The amplification of such DNA is preferably carried out using primer cyt bH 25 5'CCCCTCAAATGATATTGTCCTCA3' and primer cyt bL 5'CCATCCAACATCTCAGCATGATGAAA3', respectively.

30 This method may be carried out to distinguish mammals, birds, and fish in freshly frozen samples; or to distinguish between closely related fish, namely Atlantic salmon and brown trout; or to distinguish between domesticated animals, namely cow, pig, sheep, goat and horse; or to distinguish between poultry, namely chicken and turkey; or to distinguish between game, namely deer, moose and caribou; or to distinguish between salmon and 35 mackerel in smoked samples; or to distinguish between herring species in pickled samples; or to distinguish between salmon and tuna species in canned samples; or to

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distinguish between cod species in salted samples; or to distinguish between battered cod and chicken nuggets in partially cooked samples; or to distinguish between various birds in blood smears in slide samples; or to 5 distinguish between various mammals and birds in preserved skin samples.

The method of another mode of the invention is characterized by the steps of: isolating DNA from the sample; amplifying a defined segment of the DNA; 10 determining the nucleotide sequence of the amplified segment; and comparing that DNA sequence with a data base of DNA sequences from known species to establish the identity of the sample, which preferably includes the further steps of: first determining if there is a match 15 of the DNA sequence of the sample with any DNA sequence in the data base; and if no direct match is observed, carrying out the cladistic analysis to determine the closest species; by the steps of: extracting DNA from the genus, species, strain, sub species or sub-set; 20 amplifying and sequencing the segments of interest, and comparing the sequences with the sequence from the sample; and repeating these steps until a match is found and the identity of the sample established.

The amplification of such DNA is carried out 25 using primer cyt bH 5'CCCTCAAATGATATTGTCCTCA3' and primer cyt bL 5'CCATCCAACATCTCAGCATGATGAAA3', respectively.

In one specific feature of this invention, the polymerase chain reaction was used to amplify a 307 base 30 pair segment of the mitochondrial cytochrome-b gene from members of four species of tuna. There is intraspecific variation at this locus in each of the species. More importantly, there are differences between the four species and these genetic markers can be used to 35 determine the species identity of an individual tuna with a high degree of confidence. Thus, the method may be used to distinguish the following species of tuna by the

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presence of nucleotide at specific sites according to the following table:

5	Species	Position					
		35	62	68	89	227	260
10	Bluefin	G	T	T	T	G	C
	Bigeye	T	T	T	T	G	T
	Yellowfin	G	C	T	T	G	T
	Albacore	G	T	C/G	G	A	T

15 The DNA is preferably isolated from the sample by: homogenizing tissue for which DNA is to be isolated; extracting the homogenate; separating the phases by centrifugation; precipitating the DNA from the aqueous phase with an alcohol; collecting the DNA as a pellet; 20 and using the crude total mitochondrial and genomic DNA in the pellet. The homogenizing step preferably is carried out in an aqueous solution containing guanidinium thiocyanate, sodium citrate, sarcosyl and mercaptoethanol. The extraction step preferably is 25 carried out with a mixture of phenol: chloroform:isoamyl alcohol. The collecting of the DNA is preferably carried out by: separating the phases by centrifugation; adding an equal volume of isopropanol to the aqueous phase; cooling to -20°C; and centrifuging to pellet the DNA. 30 The DNA preferably is obtained by the steps of: washing the pellet with cold ethanol; drying the DNA pellet; and dissolving the dried pellet in a solution of Tris/HCl and EDTA.

35 Crude mitochondrial or genomic DNA may be amplified by: first chemically synthesizing two oligonucleotides that flank the defined segment of the genome to be amplified; using these oligonucleotides as primers in a series of cycles which include separating double-stranded DNA, annealing the primers to the 40 template DNA, and elongating the defined segment of DNA using a heat-stable DNA polymerase and deoxynucleotide

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triphosphate precursors; elctrophoresing the reaction mixture, staining the DNA and excising the amplified segment of DNA from the gel; preparing a single-stranded template by asymmetric amplification; removing residual 5 nucleotides, primers and buffer salts from the single-stranded DNA by centrifugal dialysis; and recovering the single-stranded DNA. The amplification reaction preferably is carried out in an aqueous solution containing Tris/HCl, magnesium chloride; 2-10 mercaptoethanol; dATP; dCTP; dGTP; dTTP; each primer; and a heat-stable, prokaryotic DNA polymerase, e.g. by about 30 to about 40 cycles of: about 92°C for about 45 seconds as the denaturation step, about 50°C for about 45 seconds as the annealing step, and about 72°C for about 15 90 seconds as the extension step. The excising of the DNA is preferably carried out by first electrophoresing the reaction mixture in a low melting agarose gel made with a Tris, sodium acetate, buffer; visualising the DNA by staining with ethidium bromide and excising the 20 amplified segment of DNA from the gel and melting in water. The single-stranded template preferably is prepared by asymmetric amplification in an aqueous solution containing Tris/HCl, magnesium chloride, 2-mercaptopethanol, dATP, dCTP, dGTP, dTTP, each primer, 25 and a heat-stable, prokaryotic DNA polymerase. The amplification is preferably carried out for about 30 to about 40 cycles of about 92°C for about 45 seconds, about 50°C for about 45 seconds, and about 72°C for about 90 seconds.

30 By yet another embodiment and feature, the DNA sequencing of the single-stranded amplified product is carried out using the dideoxy chain termination DNA sequencing procedure. The limiting primer used in the asymmetric single-stranded amplification reacting is 35 preferably used as the sequencing primer. The analysis routinely covers at least 300 nucleotides of the defined segm nt.

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Specific complementary sequences to characterize species of tuna are: part of the cytochrome-b gene of THUNNUS TUNA having the following sequence:

			* * * *	
5	AGCCAACGAA	GGCGGTCATC	ATAACTAGGA	GTAGGAGTAC 40
			* * *	
	TACTCCGATG	TTTCATGTTT	CTTTGTATAG	GTAAGAGCCG 80
	*	*	* *	
	TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATRCAGATAA 120
10		*		* *
	AGAAGAAAGA	GGCCCCGTTT	GCCTGGAGGT	TCCGGATGAG 160
	* *			*
	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG 200
		*	*	
15	GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCCA 240
		* *		
	GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC 280
	AAGTAGTGAG	CCAAAGTTT		299;
	part of the cytochrome- <u>b</u> gene of			
20	BLUE FIN			
	TUNA having	the following sequence:		
	AGCCAACGAA	GGCGGTCATC	ATAACTAGGA	GTAGGAGTAC 40
	TACTCCGATG	TTTCATGTTT	CTTTGTATAG	GTAAGAGCCG 80
			*	
	TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATACAGATAA 120
25			*	
	AGAAGAAAGA	GGCCCCGTTT	GCCTGGAGGT	TCCGGATAAG 160
	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG 200
	GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCCA 240
		*		
30	GGAATAGTCC	TGTAAGGATC	TGAGAAATAA	GGCAAAGGCC 280
	AAGTAGTGAG	CCAAAGTTT		299;
	part of the cytochrome- <u>b</u> gene of			
	YELLOW FIN TUNA			
	having the following sequence:			
	AGCCAACGAA	GGCGGTCATC	ATAACTAGGA	GTAGGAGTAC 40
35		*		
	TACTCCGATG	TTTCATGTTT	CCTTGTATAG	GTAAGAGCCG 80

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*

	TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATGCAGATAA	120
				*	
5	AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATAAG	160
	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
	GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCGA	240
	GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
	AAGTAGTGAG	CCAAAGTTT			299;

part of the part of the cytochrome-b gene of BIG
10 EYE TUNA having the following sequence:

	AGCCAACGAA	GGCGGTTCATC	ATAACTAGGA	GTAGTAGTAC	40
	TACTCCGATG	TTTCATGTTT	CTTTGTATAG	GTAAGAGCCG	80
	*			*	
15	TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATACAGATAA	120
	AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATGAG	160
	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
	*			*	
	GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGCATTGCGA	240
20	GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
	AAGTAGTGAG	CCAAAGTTT			299;

and part of the cytochrome-b gene of ALBACORE
TUNA having the following sequence:

	AGCCAACGAA	GGCGGTTCATC	ATAACTAGAA	GTAGGAGTAC	40
	*		*		
	TACTCCGATG	TTTCATGTTT	CTTTGTAGAG	GTAAGAGCCG	80
	*		*		
	TAGTAAAGGC	CTCGGCCGAT	GTGGAAGTAG	ATGCAGATAA	120
30	AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATGAG	160
	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
	*		*		
	GCGAAGGCTG	ATTCGACATC	AGGGGTATAG	TGTATTGCGA	240
	GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
35	AAGTAGTGAG	CCAAAGTTT			299.

As stated hereinabove, the oligonucleotide
primers used in the present invention have been described

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in Kocher et al. Proc. Natl. Acad. Sci. 86: 6196-6200) (1989).

5 This oligonucleotide primer is useful in the process of the present invention for many animals, e.g. mammals, birds and fish as has been shown in Table 1 above.

10 As described above, the laboratory procedures are standard PCR techniques and have been described many times by many people. (This is for the amplification and preparation of double-stranded and single-stranded DNA).

15 The sequencing protocols follow a kit provided by US Biochemical Corporation. The DNA isolation procedure is a modification of previously published methods but the modifications are slight.

20 15 The sequences provided herein are part of the non-coding sequence of the cytochrome-b genes of tuna species. This segment of the cytochrome-b protein (inferred from the complementary sequence of that determined) is identical in the four species of *Thunnus* tuna that were examined according to the process of this invention.

(iv) Brief Description of the Drawings

In the accompanying drawings,

25 Fig. 1 is a copy of the autoradiograph described in the data analysis;

Fig. 2 is a representation of the nucleotide sequence of an amplified segment; and

30 Figs. 3A, 3B and 3C, collectively designated Fig. 3, show the intra-specific variation at the cytochrome-b gene locus for each of the species tested and as described in the data analysis.

(v) Best Mode For Carrying Out Invention

Before describing the examples, some preliminary experiments will be described.

35 The most common procedure for isolating DNA uses protease digestion in the presence of the anionic detergent SDS followed by organic extraction (phenol

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and/or chloroform) and then ethanol precipitation of the DNA. This method has been very successful and high molecular weight DNA is usually obtained from fresh tissue. However, many of the specimens that one encounters in forensic work are far from fresh and meat inspectors and game wardens often have to deal with meat that is "well hung" or has been processed.

The preferred procedure for isolating "good quality" DNA from fish tissues which routinely yielded DNA capable of being amplified by PCR was a modification of a method designed to isolate RNA (see Chomczynski, P. and N. Sacchi. 1987. Single step method of RNA isolation by acid quanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.) A modification of this method has been used to prepare DNA for fingerprint analysis. (see B.J. Turner, J.E. Elder and T.F. Laughlin. 1989. DNA fingerprinting of fishes: a general method using oligonucleotide probes. *Fingerprint News* 4(4):15-16.)

Several factors had to be considered before a specific segment of DNA was selected for amplification. This region of the genome has to accept mutations sufficiently quickly that closely related organisms have different sequences, but not so rapidly that the amount of variation within a species is substantial. The length of segment had to be long enough to permit the detection of sequence differences between congeneric species, but short enough that its nucleotide sequence could be determined from a single loading of a standard DNA sequencing gel. A protein coding region of a gene was preferred because amplification and/or sequencing errors could be detected by translating the nucleotide sequence and comparing it with known amino acid sequences of the gene. A knowledge of the pattern of mutations in the gene would be an asset as would information on the structure and function of the corresponding protein, especially invariant amino acids. This would also

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facilitate comparing the nucleotide sequence from a sample with those in a data bank. Moreover, the creation of a data bank is a lengthy process and so it was desirable to take advantage of a gene whose sequence had 5 been determined in many diverse organisms. The mitochondrially encoded gene for cytochrome-b satisfies these criteria admirably.

Part of the nucleotide sequence of the gene for cytochrome-b has been determined for many vertebrates 10 including mammals, birds, and fish. Thus, a substantial data base already exists. The amino acid sequence of cytochrome-b is highly conserved but because of the degeneracy of the genetic code, the genes for cytochrome-b differ by at least a few nucleotides even in very 15 closely related species. A mitochondrially encoded gene has the additional advantage of behaving as if it were a haploid locus and therefore the problem of heterozygosity is avoided (heteroplasmy being rare in mitochondria). It is now known that one pair of primers could be used to 20 amplify a 307 basepair segment of the cytochrome-b gene from any vertebrate that was tested. The cytochrome-b gene corresponding to amino acids 33 to 134 is the segment of choice for FINS. Other regions may also be used: e.g. ribosomal RNA genes in bacteria.

25 The following are examples of the invention:

EXPERIMENT 1

FIRST PROCEDURE FOR THE EXTRACTION OF DNA

DNA is extracted from tissue (e.g. muscle, hair, blood or bone) by homogenizing the sample in 4 M 30 guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. Following homogenization, the solution is extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The phases are separated by centrifugation and to the aqueous phase 35 is added an equal volume of isopropanol. The mixture is left overnight at -20°C and then centrifuged to pellet the DNA. The DNA pellet is washed with cold (-20°C) 70%

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ethanol, dried under vacuum, dissolved in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0 and stored at 4°C. This crude total (mitochondrial and genomic) DNA extract is used as the substrate for the amplification procedure.

5 SECOND PROCEDURE FOR THE EXTRACTION OF DNA

Source and Identification of Tuna

Samples of skeletal muscle were collected from the dorsal fin area of different tuna species by fisheries officers from Canada's Department of Fisheries 10 and Oceans. The tuna were caught off southwest Nova Scotia. Samples of bluefin tuna from the Virgin Rock off Newfoundland were also examined. These samples were bagged individually, labelled, and either kept on ice or frozen at -20°C before being shipped in locked containers 15 to the Biochemistry Department at Memorial University in St. John's, Newfoundland. Full capture data including otolith number and a photo log of each sample were maintained. In addition, representative specimens of the four species of tuna examined in this study were 20 delivered whole to the laboratory where the experiments were carried out.

Electrophoretic Analysis of Muscle Proteins

Approximately 0.2 g of muscle tissue was homogenised by hand using a plastic pestle (Mandel 25 Scientific) in a 1.5 mL EPPENDORF™ tube in 1 mL of 12 mM Tris/borate buffer, pH 9.0. The homogenate was centrifuged at 12000 x g for 15 min at 4°C. The supernatant was diluted 10 fold with sterile, deionised water and approximately 0.5 µL was applied to a Pharmacia 30 isoelectric-focusing PHASTGEL™ system (pH range 3-9). After the isoelectric-focusing was completed, the gel was stained with Coomassie brilliant blue R350. Precast isoelectric-focusing gels and protein standards were obtained from Pharmacia (Canada) Ltd., Dorval, Que.

35 Briefly, 2-5g of muscle was cut into small pieces and homogenised directly at room temperature with a glass-TEFLON™ homogeniser in 10 mL of a solution

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containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. One and a half millilitres of 2 M sodium acetate, pH 4.1, was then added to the homogenate 5 and the solution was mixed with 10 mL of water-saturated phenol and 4 mL of chloroform/isoamyl alcohol (24:1, v/v). After shaking, the mixture was left on ice for 15 min and then centrifuged at 10,000 x g for 20 min at 4°C. The aqueous phase was transferred to a fresh tube 10 containing 10 mL of isopropanol. The solutions were mixed and left at -20°C for at least 1 h. Precipitated nucleic acids were obtained by centrifugation at 10,000 x g for 20 min at 4°C. The pellet was washed with cold (-20°C) 70% ethanol, dried briefly under vacuum, and 15 dissolved in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0, and stored at 4°C. This crude nucleic acid preparation was suitable for DNA amplification.

THIRD PROCEDURE FOR THE EXTRACTION OF DNA

20 Ground beef, ground port, lamb chops, chicken breasts, a frozen turkey, partially-cooked battered cod, chicken nuggets, and canned tuna and salmon were purchased from a local supermarket (in the St. John's, Newfoundland, Canada area). Fresh cod fillets, smoked salmon, smoked mackerel, pickled herring, and salt cod 25 were obtained from Fishery Products International (St. John's, Newfoundland). Other biological samples (e.g. blood, tissues not mentioned above, formalin-fixed specimens, skins, etc.) were found in the Faculty of Science, Memorial University, St. John's, Newfoundland.

30 The sample was cut into small pieces and homogenised directly at room temperature with a glass-TEFLON™ Homogeniser in a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1M 2-mercaptoethanol. For every 10 35 mL of homogenate, 1.5 mL 2M sodium acetate, pH 4.1 was added and the solution was mixed with 10 mL of Tris-saturated phenol and 4 mL of chloroform/isoamyl alcohol

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(24:1, v/v). After shaking, the mixture was left on ice for 15 min and then centrifuged at 10,000 x g for 20 min at 4°C. The aqueous phase was transferred to a fresh tube containing 10 mL of isopropanol. The solutions were 5 mixed and left at -20°C for at least one hour. Precipitated nucleic acids were obtained by centrifuging at 10,000 x g for 20 min at 4°C. The pellet was washed with cold 70% ethanol, dried briefly under vacuum, and dissolved in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0 and 10 stored at 4°C. This crude nucleic acid preparation was suitable for DNA amplification.

Synthesis of Oligonucleotide Primers

The oligonucleotide primers were synthesised using cyanoethyl phosphoramidite chemistry in the 15 Biochemistry Department, Memorial University, St. John's, Newfoundland on a MILLIGEN/BIOSEARCH_{TM} cyclone DNA synthesizer in the DMT-off mode. It was assumed that all 20 steps in the synthesis and recovery of an oligonucleotide had efficiencies of 100%. It was not necessary to purify the oligonucleotides prior to use. The primers used, cyt bH and cyt bL, were 5'CCCTCAAATGATATTGTCCTCA3' and 5'CCATCCAACATCTCAGCATGATGAAA3', respectively.

EXPERIMENT 2

AMPLIFICATION OF DEFINED SEGMENTS OF THE DNA

25 One procedure is as follows:

Two oligonucleotides, that flank the defined segment of the dna to be amplified, are chemically synthesized. These are the primers for the amplification reaction. The amplification reaction is carried out in a final 30 volume of 25 µl in microcentrifuge tubes in a solution containing 67 mM Tris/HCl; pH 8.8; 2 mM magnesium chloride; 10 mM 2-mercaptoethanol; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 10 pmoles of each primer; and 1 unit of a heat-stable, prokaryotic DNA polymerase. 35 Amplification is carried out by 30 - 40 cycles of : 92°C for 45 seconds as the denaturation step, 50°C for 45 seconds as the annealing step, and 72°C for 90 seconds as

Primer ii

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the extension step. Fifteen μ l of the reaction mixture is electrophoresed at 4°C in a 2% low melting agarose gel made with a 40 mM Tris, 30 mM sodium acetate, pH 7.4 Buffer. The DNA is visualized by staining with ethidium bromide and the amplified fragment of DNA is excised from the gel and melted in 100 μ L of water at 65°C for five minutes. The sample solution is used to prepare a single-stranded template by asymmetric amplification, except that 40 pmoles of one of the primers is used and only 0.4 pmoles of the second primer is present. Successful amplification after 30 - 40 cycles of 92°C for 45 seconds, 50°C for 45 seconds, and 72°C for 90 seconds is determined by electrophoretic analysis of 5 μ L of the reaction mixture in a 2% agarose gel. After staining with ethidium bromide, three bands are visible: a double-stranded band, a middle single-stranded band and a lower faint band containing excess primer (see Figure 1). Residual nucleotides, primers and buffer salts are removed from the single stranded product by centrifugal dialysis. The single-stranded DNA product is obtained in a final volume of approximately 60 μ L and 7 μ L is used for DNA sequencing.

Amplification of the Mitochondrial Cytochrome-b Gene

Another procedure for amplifying the mitochondrial cytochrome-b gene from a crude total cellular DNA preparation obtained from tuna muscle is as follows, namely one which was adapted from the method of Kocher et al. (see Kocher, T.D., W.K. Thomas, A. Meyer, S.V. Edwards, S. Paabo, F.X. Villablanca and A.C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci USA 86:6196-6200.) The primers were designated cyt bH and cyt bL. The nucleotide sequence of cyt bH is 5'CCCTCAGAATGATTTGTCCTCA3' and that of cyt bL is 5'CCATCCAACATCTCAGCATGATGAAA3'. The primers were synthesized by W.S. Davidson in the Department of

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Biochemistry at Memorial University, St. John's, Newfoundland on a MILLIGEN/BIOSEARCH_{TM} cyclone DNA synthesizer in the DMT-off mode. It was not necessary to purify the oligonucleotides prior to use. Double-stranded amplifications were carried out in a final volume of 25 μ l in 0.5 mL microcentrifuge tubes in a reaction cocktail containing 67 mM Tris/HCl, pH 8.8; 2 mM magnesium chloride; 10 mM 2-mercaptoethanol; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 10 pmol of each primer; and 1 unit of AMPLITAQ_{TM} DNA polymerase (Cetus, Emeryville, CA). A drop of mineral oil was added to cover the reaction mixture and 30 cycles of amplification (92°C for 45 s, 50°C for 45 s, and 72°C for 90 s) were carried out using a PERKIN-ELMER_{TM} (Irvine, CA) DNA THERMAL CYCLER_{TM}. Fifteen microlitres of the reaction mixture was electrophoresed at 4°C through a 2% NUSIEVE_{TM} (FMC BioProducts, Rockland, ME) agarose gel made up and run in a 40 mM Tris, 30 mM sodium acetate buffer titrated to pH 7.4 with HCl. The DNA was visualised by staining with ethidium bromide and the amplified fragment of DNA was excised from the gel and melted in 100 μ l of sterile, deionised water. The sample was heated to 65°C for 5 min and mixed vigorously. Five microlitres of this solution was used to prepare a single-stranded template by asymmetric amplification. The reaction was carried out in 100 μ L of the same reaction cocktail and under the same cycling conditions as those used for the double-stranded amplification (see above) except that 40 pmol of the cyt bH primer was used and only 0.4 pmol of the cyt bL primer was present. Five microlitres of the reaction mixture was sufficient to visualise double- and single-stranded DNA bands following electrophoresis through a 2% agarose gel and staining with ethidium bromide. Residual nucleotides, primers, and buffer salts were removed from the single-stranded template by centrifugal dialysis using CENTRICON_{TM}-30 filters (Amicon Ltd., Oakville, Ont.). Two millilitres of water was added to the

Purifying vi

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reaction mixture and the solution was applied to the filtration apparatus and centrifuged at 3500 x g for 20 min at 20°C. An additional 2 mL of water was added and the centrifugation repeated. Another 2 mL of water was 5 added and the centrifugation was increased 1 h until the volume in the chamber was approximately 60 µL. Single-stranded DNA template was obtained in this final volume and 7 µL was taken for DNA sequencing.

Amplification of the Mitochondrial Cytochrome-b Gene

10 A third procedure for amplifying part of the mitochondrial cytochrome-b gene from a crude nucleic acid extract obtained as described above, was as follows, namely one which was adapted from the method of Kocher et al. (see Kocher, T.D., W.K. Thomas, A. Meyer, S.V. 15 Edwards, S. Paabo, F.X. Villablanca and A.C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci USA 86:6196-6200.)

20 Double-stranded amplifications were carried out in a final volume of 25 µL in 0.5 mL microcentrifuge tubes in a reaction cocktail containing 67 mM Tris/HCl, pH 8.8; 2 mM magnesium chloride; 10 mM 2-mercaptoethanol; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 10 pmoles of cyt bH and cyt bL, and 1 unit of AMPLITAQ_{TM} DNA polymerase 25 (Cetus, Emeryville, CA). PROMEGA TAQ_{TM} polymerase from Fisher Scientific, Halifax works equally well using the buffer conditions suggested by the supplier. A drop of mineral oil was added to cover the reaction mixture and 30 cycles of amplification (92°C for 45 s, 50°C for 45 s, 30 and 72°C for 90 s) were carried out using a PERKIN-ELMER (Irvine, CA) DNA THERMAL CYCLER_{TM}. Fifteen µL of the reaction mixture were electrophoresed at 4°C through a 2% NUSIEVE_{TM} (FMC BioProducts, Rockland, ME) agarose gel made up and run in a 40 mM Tris, 30 mM sodium acetate 35 buffer titrated to pH 7.4 with HCl. The DNA was visualised by staining with ethidium bromide and the amplified fragment of DNA was excised from the gel and

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melted in 100 μ L of sterile, deionised water. The sample was heated to 65°C for five minutes and mixed vigorously. Five μ L of this solution was used to prepare a single-stranded template by asymmetric amplification. (see 5 Gyllensten, U. and H. Erlich. 1988. Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc. Natl. Acad. Sci. USA. 85:7652-7656). The reaction was carried out in 100 μ L of the same reaction cocktail 10 and under the same cycling conditions as those used for the double-stranded amplification (see above) except that 40 pmoles of the cyt bH primer was used and only 0.4 pmoles of the cytBL were present. Five μ L of the reaction were sufficient to visualise double and single- 15 stranded DNA bands following electrophoresis through a 2% agarose gel and staining with ethidium bromide. Residual nucleotides, primers and buffer salts were removed from the single-stranded template by centrifugal dialysis using CENTRICON_{TM}-30 filters (Amicon Ltd., Oakville, 20 Ont.). Two mL of water were added to the reaction mixture and the solution was applied to the filtration apparatus and centrifuged at 3,500 x g for 20 minutes at 20°C. An additional 2 mL of water were added and the centrifugation repeated. Another 2 mL of water were 25 added and the centrifugation was increased to 1 hour until the volume in the chamber was approximately 60 μ L. Single-stranded DNA template was obtained in this final volume and 7 μ L were taken for DNA sequencing.

EXAMPLE I

30 SEQUENCING REACTIONS

One procedure for DNA sequencing of 7 μ L of the single-stranded amplified product was carried out using a standard dideoxy nucleotide chain termination procedure. Ten pmoles of the limiting primer used in the 35 asymmetric single-stranded amplification reaction is used as the sequencing primer. A typical analysis routinely covers at least 300 nucleotides of the defined segment.

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Nucleotide sequencing of the single-stranded template was carried out using Sequenase and a commercial DNA sequencing kit purchased from United States Biochemical Corporation, Cleveland, Ohio. One picomole of cyt bL was used as the primer and α^{35} SdATP was used as the label. The sequencing reactions were performed according to the manufacturer's instructions except that the elongation mix was diluted 50-fold. The sequencing products were separated on 6% polyacrylamide denaturing gels. The primers amplified a 307 base pair (excluding size of primers) segment of the cytochrome-b gene and these procedures routinely allowed the sequence of 290 nucleotides of the non-coding strand to be determined.

A second procedure for nucleotide sequencing of the single-stranded template was carried out using SEQUEENASE_{TM} and a commercial DNA sequencing kit purchased from United States Biochemical Corporation, Cleveland, OH. One pmole of cyt bL was used as the sequencing primer and ³⁵SdATP was used as the label. The sequencing reactions were performed according to the manufacturer's instructions except that the elongation mix was diluted fiftyfold. The sequencing products were separated on 6% polyacrylamide denaturing gels. (see Sanger, F., S. Nicklen and R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.) The primers amplify a 307 base pair (excluding size of primers) segment of the cytochrome-b gene and these procedures routinely allowed the sequence of 290 nucleotides of the non-coding strand to be determined.

DATA ANALYSIS

The sequence of the defined segment of the DNA of the sample is then compared with the corresponding sequences from known species. If an exact fit is not observed, a cladistic analysis is carried out to predict the closest group of species to the unknown sample. DNA is then extracted from these species and subjected to

Blast?

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amplification and sequence analysis. The sequence from the test sample is now compared with these additional sequences, and the procedure is repeated until an identification is made. In many instances it may be 5 important to examine several individuals from different populations of a species in order to gauge the range of variation within that species and to determine if there is population structuring.

Comparison of Muscle Proteins

10 Isoelectric-focusing (pH range 3-9) of water-soluble muscle proteins from four tuna species yielded two groups: one characteristic of bluefin and yellowfin tunas and the other of albacore and bigeye. However, it was not possible to discriminate between individual 15 members within either group. Similar results were obtained using a narrower pH range.

Comparison of Mitochondrial Cytochrome-b Gene Sequences

20 The nucleotide sequence of a portion of the complementary strand of the mitochondrial cytochrome-b gene was obtained from 33 bluefin, 32 bigeye, 33 yellowfin, and 12 albacore tuna. A consensus sequence of this region was derived by comparing all of the 25 sequences. The numbering system is arbitrary but representative of results that could be obtained with ease from a single loading on a sequencing gel 40 cm in length. Number 296 identifies the nucleotide complementary to the first base of the codon for amino acid 33 (phenylalanine) of tuna cytochrome-b.

30 As seen in Figures 1, 2 and 3, the nucleotide sequences reveal intraspecific variation in this group of tuna. For example, six genotypes were observed in the 33 bluefin that were examined. Twenty-eight of the bluefin tuna shared an identical genotype. Four of the other 35 five genotypes differed by a single substitution from the consensus for this species, and the fifth had an additional nucleotide change. Four genotypes were observed among the 33 yellowfin examined. The most

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common genotype was shared by 28 fish. Three other yellowfin genotype was shared by 28 fish. Three other yellowfin had a genotype that differed from the common type at two positions. Two additional yellowfin each had 5 unique genotypes that differed from the common type by a single mutational event. The 12 albacore tuna gave four genotypes. Six albacore shared one genotype, four individuals shared another genotype, and two other genotypes were found in individual fish. These genotypes 10 differ from the most common albacore sequence by one nucleotide substitution. Bigeye tuna showed the most intraspecific variation. Indeed, 11 different genotypes were detected among 32 bigeye tuna tested. There were two main groupings with nine individuals in each. Four 15 bigeye shared a separate genotype as did two pairs. Another six individuals had unique genotypes. The extensive variation within this sample of bigeye suggests that there may be discrete breeding populations of bigeye tuna.

20 Although intraspecific variation was observed at the cytochrome-*b* locus in bluefin, bigeye, yellowfin, and albacore tuna, distinctive species-specific sequence differences were also uncovered in each of these four species of tuna. The data are summarised in Table 1.

25

Table 1. Nucleotides at specific sites can be used to identify tuna species.

30	Species	Position					
		35	62	68	89	227	260
	Bluefin	G	T	T	T	G	C
	Bigeye	T	T	T	T	G	T
35	Yellowfin	G	C	T	T	G	T
	Albacore	G	T	C/G	G	A	T

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For example, the 32 big ye tuna that were studied all had a thymine (T) at position 35 whereas members of the three other species had a guanine (G). Similarly, the 33 yellowfin tuna had a cytosine (C) at position 62 whereas 5 a T was present in the other species. Albacore tuna differed from all the other tuna at three sites (68, 89, and 227). The sample of 33 bluefin tuna had a C at position 260 whereas all other tuna examined had a T. These changes appear to be "fixed" in a particular 10 species, thus providing the possibility of using them as genetic markers to define individual members of that species; but this is not the case. For example, the 12 albacore had an adenine (A) at position 29. All of the bluefin and yellowfin tuna had a G at this position as 15 did 29 of 32 bigeye. However, three of the bigeye had an A at position 29. The most likely explanation is that one or more mutations have occurred at this position in the bigeye species. The pattern of nucleotide substitutions in mitochondrial cytochrome-b genes 20 predicts that this will be fairly common. A similar situation is seen at position 158 in bluefin tuna. All but one of the 33 bluefin have an A at this position whereas it is G in all members of the other species. Again, the most likely scenario to explain this 25 observation is that a mutation has occurred at this site in the lineage leading to the variant bluefin. Position 113 provides yet another example of this phenomenon. The consensus sequence at this position was designated R for purine (A or G). Bluefin and bigeye have an A at this 30 site but albacore and 30 of 33 yellowfin tuna had a G. A mutation from G to A in one lineage of yellowfin tuna could account for this change. Rather than using a particular nucleotide at a site to determine the species identify of an individual tuna, it is essential to use 35 all the information available over the entire sequence. This is a form of pattern recognition and calls for a phylogenetic analysis. The nucleotide sequence

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information provides a frame work for this type of analysis. When the nucleotide sequence of the cytochrome-b gene has been determined for a tuna of unknown species, the sequence can be fitted into the framework by using a computer program, e.g. pylogenetic analysis using parsimony (PAUP). The most likely species identity and an estimate of the reliability of the prediction can thus be calculated. This procedure also takes into account mutations in individual tuna that were not seen in this study.

None of the nucleotide substitutions resulted in a change in amino acid sequence. All but one of the substitutions are third position silent changes; the other detected in one of the bigeye is a first position C to T in a leucine codon and therefore is also silent. Most of the substitutions were transitions (i.e. the interchange of pyrimidines C to T or primers A to G). However four instances of transitions (i.e. a change from a purine to a pyrimidine or vice versa) were noted at positions 32, 35, 68, and 89. This latter type of nucleotide substitution accounted for approximately 20% of the changes observed among the tuna cytochrome-b genes. This pattern of nucleotide substitution has been seen in other comparisons of fish cytochrome-b genes.

25 Nucleotide Sequence Analysis

No single nucleotide sequence of the cytochrome-b gene is diagnostic of a particular species. This is because of the nature of mutations in mitochondrial genes coding for proteins and intraspecific variation. This must be taken into account in FINS as it will be impossible to sample enough individuals from all the species that one may wish to have in a data bank to cover all the genetic variation within a species. Therefore, the procedure that is used to overcome this potential problem relies on a phylogenetic analysis. This is a form of pattern recognition and makes use of all the information in a sequence. Fortunately, this field of

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r search is well established in molecular evolutionary studies and computer programs are available (e.g. PAUP). (see Swofford, D.L. 1989. PAUP: phylogenetic analysis using parsimony, Version 3.0b. Illinois Natural History Survey, Champaign, IL.) This procedure also allows reversions and new mutations to be taken into account. Moreover, if the species to which the specimen belongs is not represented in the data base, the phylogenetic analysis will reveal the most closely related species.

5 At this point it would be possible to obtain additional standard species and to expand this section of the data bank. It has been our experience that the amount of intra-specific variation does not preclude the identification of even closely related species. However,

10 15 there may be some very closely related organisms, that are regarded as separate species based on morphological characteristics, that are not genetically distinguishable at the molecular level. In these instances, it will not be possible to use FINS or any other biochemical genetic

20 25 method to distinguish members of these species from one another.

EXAMPLE II

The DNA extracted from samples was examined and it was found that the nucleic acids in the final preparation were usually less than five to seven kilobase pairs in length (estimated by ethidium bromide staining after agarose gel electrophoresis). The protocol outlined above is easily scaled down for small amounts of sample. It is rapid and amplifiable DNA was obtained

30 35 from a wide variety of samples including processed meats. This is shown below in Table 2.

The FINS procedure was successful in determining the species identify of biological samples that had been processed in the following ways.

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Table 2.

<u>Process</u>	<u>Samples Tested</u>
Freshly frozen	Many mammals, birds, fish
Ground meat	Beef, pork
5 Smoked	Salmon, mackerel
Pickled	Herring
Canned	Salmon, tuna species
Salted	Cod
Partially cooked	Battered cod, chicken nuggets
10 Formalin fixed	Salmonids
Ethanol preservation	Salmonids
Blood smears on slides	Birds
Preserved skins	Mammals, birds

15 The following example illustrates the use of the procedure outlined above.

EXAMPLE III

Four commercially-important tuna species in the genus *Thunnus* are caught off the east coast of Canada. The harvest of bluefin tuna is regulated, whereas that of 20 bigeye, yellowfin, or albacore is not. However, enforcement of the regulations governing the bluefin tuna fishery was difficult because of the close genetic relationships among these species and the ease with which morphological characters may be removed once a fish has 25 been landed. Furthermore, analysis of muscle proteins using sensitive methods, e.g. isoelectric focusing could not distinguish bluefin tuna from yellowfin tuna.

DNA was isolated from muscle samples from 30 commercially caught tuna that had been identified by officers from the Canadian Department of Fisheries and Oceans. The polymerase chain reaction procedure which has been described above and which has also been described in U.S. Patent 4,683,195, the content of which are herein incorporated by reference, was used to amplify 35 a region of the cytochrome-b gene from members of these species. The nucleotide sequence of the amplified segment was determined by the dideoxy chain termination

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procedure. An example of these results is shown in Figure 2. The corresponding sequences were compared for 33 bluefin, 33 yellowfin, 32 bigeye, and 12 albacore. Intra-specific variation was observed at this locus in 5 each of the species and this is shown in Figure 3. More importantly, there are fixed differences between each of these four species. For example: position 29 in Figure 10 3 shows that albacore tuna have an A at this position, whereas in the other species of tuna it is a G; position 35 in Figure 3 shows that big eye tuna have a T at this position, whereas in the other species of tuna it is a G; position 62 in Figure 3 shows that yellow fin tuna have a C at this position, whereas in the other species of tuna it is a T; position 68 in Figure 3 shows that 15 albacore tuna have a G at this position, whereas in the other species of tuna it is a T; position 89 in Figure 3 shows that albacore tuna have a G at this position, whereas in the other species of tuna it is a T; position 218 in Figure 3 shows that bluefin tuna have an A at this 20 position, whereas in the other species of tuna it is a G; position 227 in Figure 3 shows that albacore tuna have an A at this position, whereas in the other species of tuna it is a G; and position 260 in Figure 3 shows that bluefin tuna have a C at this position, whereas in the other species of tuna it is a T. This type of 25 species-specific genetic marker can be used to determine unambiguously the species identity of an individual tuna.

Markers have been identified that can readily distinguish between other closely related fish (e.g. 30 Atlantic salmon and brown trout); poultry (e.g. chicken and turkey); domesticated mammals (e.g. cow, pig, sheep, goat, horse); and game (e.g. deer, moose, and caribou).

(vi) Way In Which The Invention Is Capable of
Exploitation in Industry
35 The advantages and utility of the direct sequence analysis of fragments of DNA amplified via the polymerase chain reaction has been established by the present

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invention. A very small amount of DNA is required initially and the DNA does not have to be highly purified or of high molecular weight.

The present invention uses a pair of primers that 5 correspond to highly conserved regions of the mitochondrial cytochrome-b genes of vertebrates. These primers, in conjunction with the polymerase chain reaction, were used to amplify a 307 base pair segment of the mitochondrial genome and to determine the sequence 10 of this gene from individual tuna. There are several advantages for using this mitochondrial gene for comparing closely related species. The amino acid sequences of cytochrome-b from several diverse organisms have been determined and a consensus sequence showing 15 invariant amino acid positions has been constructed. This serves as a guide to verify that the nucleotide sequence obtained in a forensic experiment is actually that of a cytochrome-b gene. In addition, the pattern of nucleotide substitutions has been determined for many 20 species of mammals, birds, and fishes. Within a particular species and also between closely related species, transitions are more common than transversions and most substitutions occur at synonymous sites such that the amino acid sequence of the protein is not 25 altered.

The principal advantage of FINS is that FINS is a rapid, accurate and reproducible procedure that is based on established techniques. It is not subject to operator bias and can be performed independently in any 30 lab equipped to carry out standard molecular biology. Thus, an analysis could be confirmed in any other diagnostic lab. FINS produces genetic evidence and this should be acceptable to the legal system. Court cases that have previously been hampered by the lack of genetic 35 evidence can now proceed. For example, FINS allows the discrimination between bluefin tuna (a regulated species) and yellowfin tuna (an unregulated species) whereas this

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was not achieved by protein electrophoretic procedures. FINS is a rapid, reliable and reproducible procedure that is based on established techniques. Therefore, FINS fills the need for a reliable method for determining the 5 biological identity of a specimen when this is not possible by conventional means.

The keys to identifying different species normally rely heavily on morphological characteristics. However, when an animal has been killed for food or 10 sport, these markers are often destroyed or intentionally removed from the animal. This presents a problem for government agencies who are involved in determining the species origin of an animal or products derived from it in order to enforce conservation and/or health-related 15 regulations. The problem is compounded if the meat of the animal has been processed in any way. The Forensically Informative Nucleotide Sequencing procedure (FINS) overcomes these problems. First, DNA is isolated from a wide range of biological samples including 20 processed foods (e.g. canned, partially cooked, pickled, salted, or smoked) using traditional methods. Second, a specific segment of DNA is amplified using the polymerase chain reaction. Third, the nucleotide sequence of the 25 amplified segment of DNA is determined. Fourth, this nucleotide sequence is subjected to a phylogenetic analysis using a data base and the most closely related species is identified.

The area of the gene which is operative for the method of this invention is the cytochrome-b gene. When 30 used in the method of the present invention the PCR procedure amplifies only is part of the cytochrome-b gene. In the present invention, the non-coding strand of the gene is used to do the analysis in tuna. The protein sequence of the cytochrome-b gene product from tuna is a 35 novel sequence and forms part of the present invention.

The procedure of the present invention examined the DNA sequences of the cytochrome-b genes of bluefin,

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bigeye, yellowfin, and albacore. The amount of intraspecific variation that was detected in these tuna, particularly bigeye, was quite startling and served to illustrate the resolving power of this procedure.

5 Although variation is apparent within the *Thunnus* tuna species, there are, more importantly, distinct differences which characterise each of the four species. These species-specific genetic markers make it possible to use this procedure and a phylogenetic analysis to 10 determine unambiguously the species identity of an individual tuna. The methodology described above should prove very useful for enforcing the regulations governing the bluefin tuna fishery off Canada's east coast. The methodology is also readily applicable to other 15 enforcement and forensic problems.

INFORMATION FOR SEQ. I.D. NO. 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 299 nucleotides

(B) TYPE: nucleotide

20 (C) TOPOLOGY: paired linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ. I.D. NO: 1: complementary sequence of the part of the cytochrome-b gene of THUNNUS TUNA

25	AGCCAACGAA	GGCGGTTCATC	ATAACTAGGA	GTAGGAGTAC	40
			*	*	***
20	TACTCCGATG	TTTCATGTTT	CTTTGTATAG	GTAAGAGCCG	80
	*	*	*	*	
25	TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATRCAGATAA	120
	*			*	*
30	AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATGAG	160
	*	*		*	
35	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
	*		*		
	GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCGA	240

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* *

GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
AAGTAGTGAG	CCAAAGTTT			299.

INFORMATION FOR SEQ. I.D. NO. 2

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 299 nucleotides

(B) TYPE: nucleotide

(C) TOPOLOGY: paired linear

(ii) MOLECULE TYPE: DNA

10 (iii) SEQUENCE DESCRIPTION: SEQ. I.D. NO: 2:
complementary sequence of the part of the cytochrome-b
gene of BLUE FIN TUNA

AGCCAACGAA	GGCGGTACATC	ATAACTAGGA	GTAGGAGTAC	40
TACTCCGATG	TTTCATGTTT	CCTTGTATAG	GTAAGAGCCG	80

15

TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATACAGATAA	120
------------	------------	------------	------------	-----

*

AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATAAG	160
TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200

20

GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCGA	240
------------	------------	------------	------------	-----

*

GGAATAGTCC	TGTAAGGATC	TGAGAAATAA	GGCAAAGGCC	280
AAGTAGTGAG	CCAAAGTTT			299.

INFORMATION FOR SEQ. I.D. NO. 3

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 299 nucleotides

(B) TYPE: nucleotide

(C) TOPOLOGY: paired linear

(ii) MOLECULE TYPE: DNA

30 (iii) SEQUENCE DESCRIPTION: SEQ. I.D. NO: 3:
complementary sequence of the part of the cytochrome-b
gene of YELLOW FIN TUNA

AGCCAACGAA	GGCGGTACATC	ATAACTAGGA	GTAGGAGTAC	40
------------	-------------	------------	------------	----

*

35 TACTCCGATG	TTTCATGTTT	CCTTGTATAG	GTAAGAGCCG	80
---------------	------------	------------	------------	----

*

TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATGCAGATAA	120
------------	------------	------------	------------	-----

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*

AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATAAG	160
TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCGA	240
5 GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
AAGTAGTGAG	CCAAAGTTT			299.

INFORMATION FOR SEQ. I.D. NO. 4

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 299 nucleotides

10 (B) TYPE: nucleotide

(C) TOPOLOGY: paired linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ. I.D. NO: 4: complementary sequence of the part of the cytochrome-b gene of BIG EYE TUNA

*

AGCCAACGAA	GGCGGTCATC	ATAACTAGGA	GTAGTAGTAC	40
TACTCCGATG	TTTCATGTTT	CTTTGTATAG	GTAAGAGCCG	80
20 TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATACAGATAA	120
AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATGAG	160
TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
25 GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGCATTGCGA	240
GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
AAGTAGTGAG	CCAAAGTTT			299.

INFORMATION FOR SEQ. I.D. NO. 5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 299 nucleotides

30 (B) TYPE: nucleotide

(C) TOPOLOGY: paired linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ. I.D. NO: 5: complementary sequence of the part of the cytochrome-b gene of ALBACORE TUNA

*

AGCCAACGAA	GGCGGTCATC	ATAACTAGAA	GTAGGAGTAC	40
------------	------------	------------	------------	----

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	TACTCCGATG	TTTCATGTTT	CTTTGTAGAG	GTAAGAGCCG	80
	*		*	*	
5	TAGTAAAGGC	CTCGGCCGAT	GTGGAAGTAG	ATGCAGATAA	120
	AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATGAG	160
	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
			*		
	GCGAAGGCTG	ATTCGACATC	AGGGGTATAG	TGTATTGCGA	240
	GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
10	AAGTAGTGAG	CCAAAGTTT			299.

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CLAIMS

1. In a method for the determination of the genus, and then the species, and/or strain, and/or sub species and/or sub-set of sample of a eukaryote or a prokaryote 5 organism, characterized by the steps of: isolating DNA from said sample; amplifying a defined segment of said DNA; determining the nucleotide sequence of said amplified segment; comparing that DNA sequence with a data base of DNA sequences from known species; and 10 carrying out a cladistic analysis of these sequence data, to establish the identity of said sample.
2. The method of claim 1 characterized in that said organism is a mammal, a bird, a reptile, an amphibian, a fish or an invertebrate.
- 15 3. The method of claim 2 characterized by the further steps of: first determining if there is a match of the sample's DNA sequence of said sample with any DNA sequence in the data base; and if no direct match is observed, carrying out said cladistic analysis to 20 determine the closest species; by the steps of: extracting DNA from said genus, species strain species or sub-sets; amplifying and sequencing the segments of interest and comparing said sequence with the sequence from said sample; and repeating these steps until a match 25 is found and the identity of said sample established.
4. The method of claims 1, 2 or 3 characterized in that the amplification of said DNA is carried out using primer cyt bH 5'CCCTCTAAATGATATTGTCTCA3' and primer cyt bL 5'CCATCCAACATCTCAGCATGATGAAA3', respectively.
- 30 5. The method of claims 1, 2 or 3 characterized in that said method is carried out to distinguish mammals, birds, and fish in freshly frozen samples; or to distinguish between closely related fish, namely Atlantic salmon and brown trout; or to distinguish between domesticated 35 animals, namely cow, pig, sheep, goat and horse; or to distinguish between poultry, namely chicken and turkey; or to distinguish between game, namely deer, moose and

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caribou; or to distinguish between salmon and mackerel in smoked samples; or to distinguish between herring species in pickled samples; or to distinguish between salmon and tuna species in canned samples; or to distinguish between 5 cod species in salted samples; or to distinguish between battered cod and chicken nuggets in partially cooked samples; or to distinguish between various birds in blood smears in side samples; or to distinguish between various mammals and birds in preserved skin samples.

10 6. A method for the determination of the species origin of a sample of an organism and/or the population identity of a said organism, which is characterized by the steps of: isolating DNA from said sample; amplifying a defined segment of said DNA; determining the nucleotide sequence 15 of said amplified segment; and comparing that DNA sequence with a data base of DNA sequences from known species to establish the identity of said sample.

7. The method of claim 6 characterized by including the further steps of: first determining if there is a match 20 of the DNA sequence of said sample with any DNA sequence in the data base; and if no direct match is observed, carrying out said cladistic analysis to determine the closest species by the steps of: extracting DNA from said genus, species, strain, sub species or sub-set; 25 amplifying and sequencing the segments of interest, and comparing said sequences with the sequence from the sample; and repeating these steps until a match is found and the identity of the sample established.

8. The method of claim 6 characterized in that the 30 amplification of said DNA is carried out using primer cyt bH 5'CCCTCTAAATGATATTGTCCTCA3' and primer cyt bL 5'CCATCCAACATCTCAGCATGATGAAA3', respectively.

9. The method of claim 8 characterized in that said 35 method is carried out to distinguish the following species of tuna by the presence of nucleotide at specific sites according to the following table:

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Position

Species	35	62	68	89	227	260	
5							
Bluefin	G	T	T	T	G	C	
Bigeye	T	T	T	T	G	T	
Yellowfin	G	C	T	T	G	T	
10	Albacore	G	T	C/G	G	A	T

10. The method of claim 6 characterized in that said DNA is isolated from said sample by homogenizing tissue from which DNA is to be isolated; extracting the homogenate; separating the phases by centrifugation; precipitating the DNA from the aqueous phase with an alcohol; collecting the DNA as a pellet; and using the crude total mitochondrial and genomic DNA in the pellet.

15. The method of claim 10 characterized in that said homogenizing step is carried out in an aqueous solution containing guanidinium thiocyanate, sodium citrate, sarcosyl and mercaptoethanol.

20. The method of claim 10 characterized in that said extraction step is carried out with a mixture of phenol: chloroform:isoamyl alcohol.

25. The method of claim 10 characterized in that the collecting of said DNA is carried out by separating the phases by centrifugation, adding an equal volume of isopropanol to the aqueous phase, cooling to -20°C and centrifuging to pellet the DNA.

30. The method of claim 10 characterized in that the DNA is obtained by the steps of washing the pellet with cold ethanol, drying the DNA pellet, and dissolving the dried pellet in a solution of Tris/HCl and EDTA.

35. The method of claim 6 characterized in that crude mitochondrial or genomic DNA is amplified by first chemically synthesizing two oligonucleotides that flank the defined segment of the genome to be amplified; using these oligonucleotides as primers in a series of cycles which include separating double-stranded DNA, annealing the primers to the template DNA, and elongating the

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defined segment of DNA using a heat-stable DNA polymerase and deoxynucleotide triphosphate precursors; electrophoresing the reaction mixture, staining the DNA and excising the amplified segment of DNA from the gel;

5 preparing a single-stranded template by asymmetric amplification; removing residual nucleotides, primers and buffer salts from the single-stranded DNA by centrifugal dialysis; and recovering the single-stranded DNA.

16. The method of claim 15 characterized in that said

10 amplification reaction is carried out in an aqueous solution containing Tris/HCl, magnesium chloride, 2-mercaptoethanol, dATP, dCTP, dGTP, dTTP, each primer, and a heat-stable, prokaryotic DNA polymerase.

17. The method of claim 15 characterized in that said

15 amplification is carried out by about 30 - about 40 cycles of: about 92°C for about 45 seconds as the denaturation step, about 50°C for about 45 seconds as the annealing step, and about 72°C for about 90 seconds as the extension step.

20 18. The method of claim 15 characterized in that said excising of said DNA is carried out by first electrophoresing the reaction mixture in a low melting agarose gel made with a Tris, sodium acetate, buffer; visualising the DNA by staining with ethidium bromide and

25 excising the amplified segment of DNA from the gel and melting in water.

19. The method of claim 15 characterized in that the single-stranded template is prepared by asymmetric amplification in an aqueous solution containing Tris/HCl,

30 magnesium chloride, 2-mercaptoethanol, dATP, dCTP, dGTP, dTTP, each primer, and a heat-stable, prokaryotic DNA polymerase.

20. The method of claims 1 or 6 characterized in that

DNA sequencing of the single-stranded amplified product

35 is carried out using the dideoxy chain termination DNA sequencing procedure.

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21. The method of claim 20 characterized in that the limiting primer used in the asymmetric single-stranded amplification reacting is used as the sequencing primer.

22. The method of claim 20 characterized in that the analysis routinely covers at least 299 nucleotides of the defined segment.

23. Complementary sequence of the part of the cytochrome-b gene of THUNNUS TUNA having the following sequence:

10	AGCCAACGAA	GGCGGTCATC	ATAACTAGGA	GTAGGAGTAC	40
			*	*	*
15	TACTCCGATG	TTTCATGTTT	CTTTGTATAG	GTAAGAGCCG	80
	*	*	*	*	
20	TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATRCAGATAA	120
	*			*	*
25	AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATGAG	160
	*	*		*	
30	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
	*		*		
35	GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCGA	240
		*	*		
	GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
	AAGTAGTGAG	CCAAAGTTT			299.
40	24.	Complementary sequence as claimed in claim 23	comprising the part of the cytochrome- <u>b</u> gene of BLUE FIN	TUNA having the following sequence:	
45	AGCCAACGAA	GGCGGTCATC	ATAACTAGGA	GTAGGAGTAC	40
50	TACTCCGATG	TTTCATGTTT	CTTTGTATAG	GTAAGAGCCG	80
55	TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATACAGATAA	120
60	AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATAAG	160
65	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
70	GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCGA	240

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*

GGAATAGTCC	TGTAAGGATC	TGAGAAATAA	GGCAAAGGCC	280
AAGTAGTGAG	CCAAAGTTT			299.

25. Complementary sequence as claimed in claim 23
 5 comprising of the part of the cytochrome-b gene of
 YELLOW FIN TUNA having the following sequence:

AGCCAACGAA	GGCGGTACATC	ATAACTAGGA	GTAGGAGTAC	40
------------	-------------	------------	------------	----

*

TACTCCGATG	TTTCATGTTT	CCTTGTATAG	GTAAGAGCCG	80
------------	------------	------------	------------	----

10

*

TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATGCAGATAA	120
------------	------------	------------	------------	-----

*

AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATAAG	160
------------	------------	------------	------------	-----

TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
------------	------------	------------	------------	-----

15

GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGTATTGCGA	240
------------	------------	------------	------------	-----

GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
------------	------------	------------	------------	-----

AAGTAGTGAG	CCAAAGTTT			299.
------------	-----------	--	--	------

26. Complementary sequence as claimed in claim 23
 comprising the part of the cytochrome-b gene of BIG EYE
 20 TUNA having the following sequence:

*

AGCCAACGAA	GGCGGTACATC	ATAACTAGGA	GTAGTAGTAC	40
------------	-------------	------------	------------	----

TACTCCGATG	TTTCATGTTT	CTTTGTATAG	GTAAGAGCCG	80
------------	------------	------------	------------	----

*

TAGTAAAGTC	CTCGGCCGAT	GTGGAAGTAG	ATACAGATAA	120
------------	------------	------------	------------	-----

AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATGAG	160
------------	------------	------------	------------	-----

TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
------------	------------	------------	------------	-----

*

GCGAAGGCTG	ATTCGACATC	AGGGGTGTAG	TGCATTGCGA	240
------------	------------	------------	------------	-----

30 GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
---------------	------------	------------	------------	-----

AAGTAGTGAG	CCAAAGTTT			299.
------------	-----------	--	--	------

27. Complementary sequence as claimed in claim 23
 comprising the part of the cytochrome-b gene of ALBACORE
 TUNA having the following sequence:

	AGCCAACGAA	GGCGGTCATC	ATAACTAGAA	GTAGGAGTAC	40
			*		
	TACTCCGATG	TTTCATGTTT	CTTTGTAGAG	GTAAGAGCCG	80
5	*		*		
	AGAAGAAAGA	GGCCCCGTTT	GCGTGGAGGT	TCCGGATGAG	160
	TCAACCGAAG	TTGACATCTC	GGCAAATGTG	GGCTACTGAG	200
			*		
10	GCGAAGGCTG	ATTCGACATC	AGGGGTATAG	TGTATTGCGA	240
	GGAATAGTCC	TGTAAGGATT	TGAGAAATAA	GGCAAAGGCC	280
	AACTAGTGAG	CCAAAGTTT			299.
15	28.	A kit comprising a carrier; compartments within said carrier to receive one container containing DNA from a defined eukaryote or prokaryote organism; a second container containing an amplified segment of the DNA from said defined organism; and a third container containing DNA sequences for a plurality of genera, species, and/or strain, and/or sub species and/or sub-set, or DNA derived therefrom.			
20	29.	The kit of claim 28 wherein the organism is a mammal, a bird, a reptile, an amphibian, a fish or an invertebrate.			
25	30.	A kit comprising a carrier; compartments within said carrier to receive one container containing DNA from a defined organism; a second container containing an amplified segment of the DNA from the defined organism; and a third container containing DNA sequences for a plurality of species or DNA derived therefrom.			

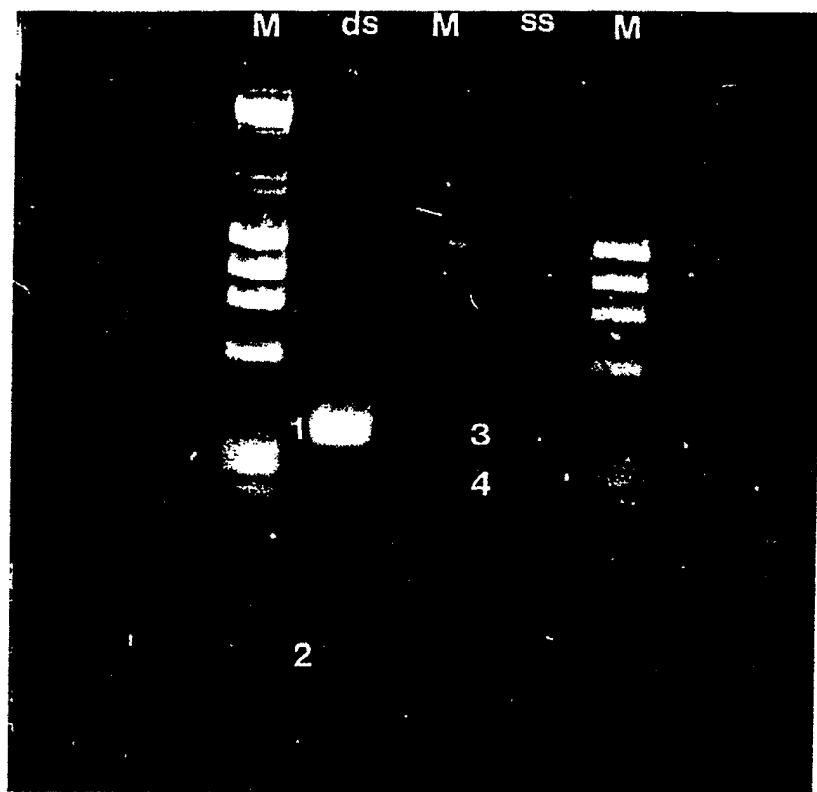


FIG. I

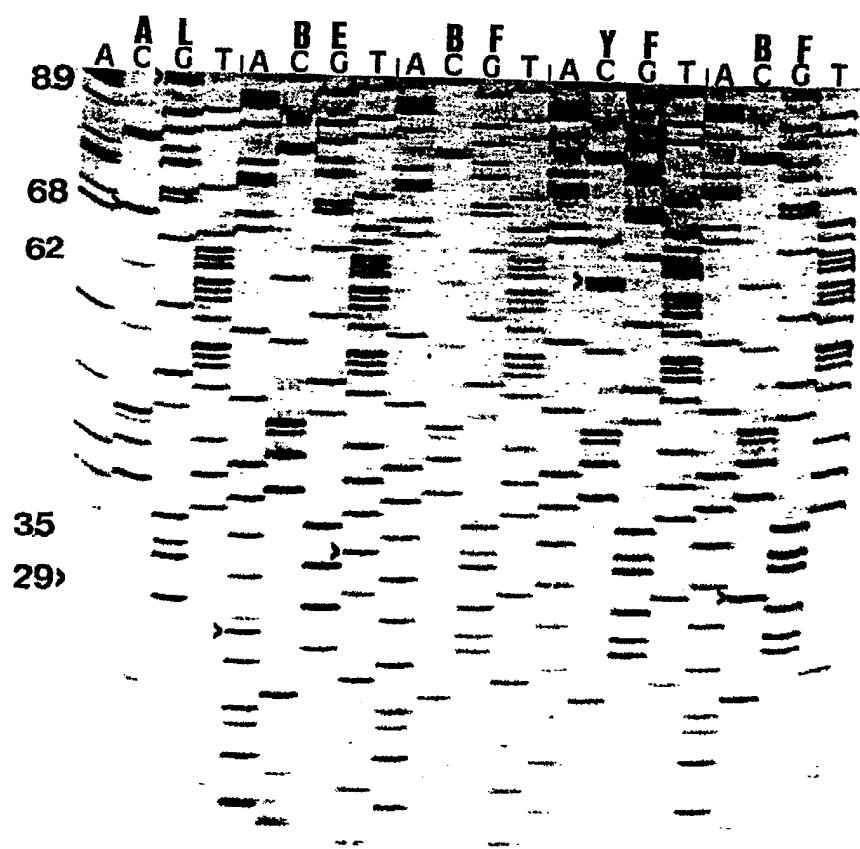


FIG. 2

60

BF (28)	AGCCAACGAAGGCGGT	CATCATAACTAGGAGTAGGAGT	ACTACTCCGATGTT	CATGTT
BF (1)	-----	-----	-----	-----
BF (1)	-----	-----	-----	-----
BF (1)	-----	-----	-----	-----
BF (1)	-----	-----	-----	-----
YF (28)	-----	-----	-----	-----
YF (3)	-----	-----	-----	-----
YF (1)	-----	-----	-----	-----
YF (1)	-----	-----	-----	-----
BE (9)	-----	a	-----	-----
BE (9)	-----	t	-----	-----
BE (4)	-----	t	-----	-----
BE (2)	-----	t	-----	-----
BE (2)	-----	a	-----	-----
BE (1)	-----	-----	-----	-----
BE (1)	-----	g	-----	-----
BE (1)	-----	-----	at	-----
BE (1)	-----	-----	a	-----
BE (1)	-----	-----	g	-----
BE (1)	-----	-----	at	-----
AL (6)	-----	A	-----	-----
AL (4)	-----	A	-----	-----
AL (1)	-----	A	-----	-----
AL (1)	-----	A	-----	-----

120

BF (28)	CTTTGTATAGGTAAGAGCCGT	AGTAAAGTCCTCGGCCGATGTGGAAGTAGATRCAGATAA	-----	-----
BF (1)	-----	-----	A	-----
BF (1)	-----	-----	-----	A
BF (1)	-----	-----	-----	-----
BF (1)	-----	-----	-----	A
BF (1)	-----	-----	-----	-----
YF (28)	C	-----	-----	G
YF (3)	C	-----	-----	-----
YF (1)	C	-----	-----	G
YF (1)	C	-----	-----	G
BE (9)	-----	-----	A	-----
BE (9)	-----	-----	A	-----
BE (4)	-----	-----	A	-----
BE (2)	-----	-----	A	-----
BE (2)	-----	-----	A	-----
BE (1)	-----	-----	A	-----
BE (1)	-----	-----	A	-----
BE (1)	-----	-----	A	-----
BE (1)	-----	-----	A	-----
AL (6)	G	-----	G	-----
AL (4)	C	-----	G	-----
AL (1)	G	-----	G	-----
AL (1)	G	-----	G	-----

FIG.3A

180

BF (28) AGAAGAAAAGAGGCCCGTTGCGTGAGCTTCCGGATGAGTCACCGAAGTTGACATCTC
 BF (1) ----- A
 BF (1) ----- A
 BF (1) ----- A
 BF (1) ----- G
 BF (1) ----- A - g

YF (28) -----
 YF (3) -----
 YF (1) -----
 YF (1) ----- d

BE (9) -----
 BE (9) -----
 BE (4) -----
 BE (2) -----
 BE (2) -----
 BE (1) ----- a
 BE (1) ----- a

AL (6) -----
 AL (4) -----
 AL (1) -----
 AL (1) -----

240

GGCAAATGTGGCTACTGAGGCGAAGGCTGATTGACATCAGGGGTGTAGTGTATTGCAG
 BF (28) -----
 BF (1) -----

YF (28) -----
 YF (3) -----
 YF (1) -----
 YF (1) -----

BE (9) ----- c
 BE (9) -----
 BE (4) -----
 BE (2) -----
 BE (2) ----- c
 BE (1) -----
 BE (1) ----- c
 BE (1) -----
 BE (1) ----- c
 BE (1) ----- c

AL (6) ----- a
 AL (4) ----- a
 AL (1) ----- a
 AL (1) ----- a

FIG.3B

300

 * *
GGAATAGTCCTGTAAGGATTGAGAAATAAGGCAAAGGCCAAGTAGTGAGCCAAAGTT
BF { 28 } -----C-----
BF { 1 } -----C-----
YF { 28 } -----
YF { 3 } -----
YF { 1 } -----
YF { 1 } -----
BE { 9 } -----
BE { 9 } -----
BE { 4 } -----
BE { 2 } -----
BE { 2 } -----
BE { 1 } -----
AL { 6 } -----
AL { 4 } -----
AL { 1 } -----
AL { 1 } -----g-----

FIG.3C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 91/00345

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 C12Q1/68

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.C1. 5	C12Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

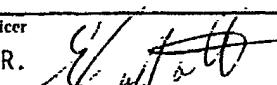
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	JOURNAL OF GENERAL MICROBIOLOGY vol. 136, no. 9, 18 September 1990, READING, UK pages 1915 - 1920; T. ROGALL ET AL.: 'Differentiation of Mycobacterium species by direct sequencing of amplified DNA' see the whole document, esp. p.1915, right column, lines 3 to 32 ---	1,6,7
Y	JOURNAL OF CLINICAL MICROBIOLOGY vol. 28, no. 9, September 1990, WASHINGTON, D.C., USA pages 1942 - 1946; K.H. WILSON ET AL.: 'Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction' see the whole document, esp. the abstract ---	1,6,7

¹⁰ Special categories of cited documents :

- ^{"A"} document defining the general state of the art which is not considered to be of particular relevance
- ^{"E"} earlier document but published on or after the international filing date
- ^{"I,"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- ^{"O"} document referring to an oral disclosure, use, exhibition or other means
- ^{"P"} document published prior to the international filing date but later than the priority date claimed

^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention^{"X"} document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step^{"Y"} document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.^{"&"} document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 DECEMBER 1991	07.01.92
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer LUZZATTO E.R. 

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, August 1989, WASHINGTON US pages 6196 - 6200; T.D.KOCHER ET AL.: 'Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers' cited in the application see the whole document</p> <p>---</p>	1-3,5-7
Y	<p>EP,A,0 120 658 (J.A.WEBSTER) 3 October 1984 cited in the application see page 3, line 21 - page 23, line 14; claims</p> <p>---</p>	1-3,5-7
A	<p>AMERICAN ZOOLOGIST vol. 28, no. 4, December 1988, CHARLOTTESVILLE,USA page 35A; A.MEYER ET AL.: 'Mitochondrial genes amplified via the polymerase chain reaction: inferences for the evolution of cichlid fishes from mitochondrial gene sequences' cited in the application see the abstract n° 177</p> <p>---</p>	1,2,5
A	<p>EP,A,0 335 633 (INTEGRATED GENETICS, INC.) 4 October 1989 see the whole document, esp. column 2, line 41-column 4, line 17</p> <p>---</p>	1
P,X	<p>CANADIAN JOURNAL OF FISHERIES AND AQUATIC SCIENCES vol. 48, no. 2, 1991, OTTAWA,CA pages 309 - 317; S.E.BARTLETT ET AL.: 'Identification of Thunnus tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome b genes' cited in the application see the whole document</p> <p>---</p>	1-30

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. CA 9100345
SA 51344

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 06/12/91

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
EP-A-0120658	03-10-84		CA-A- 1221298 JP-T- 60500895 WO-A- 8403715 WO-A- 8403716	05-05-87 20-06-85 27-09-84 27-09-84
EP-A-0335633	04-10-89		JP-A-	2150300